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81. VARIATIONS IN THE SOLIDS-NOT-FAT CONTENT OF MILK

III. THE LACTATION YIELD AND FACTORS AFFECTING IT

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(With 1 Chart.)

THE LACTATION YIELD OF MILK, FAT AND SOLIDS-NOT-FAT.

THE yield of milk associated with one calving is usually termed the "lactation yield," and this provides a convenient accounting period for calculating or expressing the general productivity of a cow. Owing to the slight variations which exist among different societies and countries as to what constitutes a lactation period, it is necessary to note the bases used in the present work. The lactation yields of milk are the actual quantities yielded by cows from the fifth day after calving till they become dry.

For the estimation of fat percentage, samples of milk were tested on three consecutive days each month and the fat content of all samples collected from the eleventh day after calving till the cows became dry were utilised for the calculation of an average lactation fat percentage. This average was obtained by weighting the fat percentage of each sample according to the yield of milk from which the sample was taken.

For the estimation of the percentage of solids in the fat-free milk, samples were tested on one day each month, and the only figures used for the calculation of the average "lactation period" value were the eight monthly tests following the eleventh day after calving. This limitation in the length of lactation periods may not be desirable for all purposes, but the object in view here was to eliminate the variations in solids-not-fat which are associated with early and late lactation and to secure figures particularly suited to the study of other causes of variation. When calculating the mean lactation value for the concentration of solids in the fat-free milk, the monthly percentages were averaged and it was not considered necessary to weight them according to the yield of milk. This direct averaging of percentages, of course, involves a slight error in the mean, but the concentration of morning's and evening's milk is so similar that little or no loss of accuracy occurs as a result of using the direct mean.

THE EFFECT OF AGE ON MILK SECRETION.

The effect of age of cows on the lactation yield, fat content and solids-not-fat content was studied. The method of grouping the lactation records was to select all the cows in which the first and second lactations were available and to

calculate the mean lactation yield with each calf. Thus the difference between first and second lactations could be found. Next, all cows in which the second and third lactation records were known were treated similarly, and by a continuation of this classification with cows of each succeeding age, changes in secretion were traced from the first to the ninth calf. The number of records available for each group average became smaller as age advanced, thereby yielding less reliable results. The grouping of the records in this manner does not produce a continuous curve, but this was obtained by commencing with the curve produced by first and second calvers and adding to this the differences between each succeeding pair of lactations.

Table I. *The variation, due to age of cow, of* (*a*) *milk yield,* (*b*) *fat percentage,* (*c*) *percentage of solids in the fat-free milk.*

	Number of lactation								
	1	2	3	4	5	6	7	8	9
Number of animals	67	67	49	34	21	12	9	4	3
(*a*) Milk yield:									
lb.	6654	7761	8879	9564	10098	10105	10401	9408	7428
Relative yields	100	116·6	133·4	143·7	151·8	151·9	156·3	141·4	111·6
Increments and standard errors	—	1107 ± 191	1118 ± 275	685 ± 270	534 ± 370	—	—	—	—
(*b*) Concentration of fat:									
Percentage	3·95	3·90	3·90	3·84	3·79	3·70	3·74	3·67	3·74
Relative content	100	98·7	98·7	97·2	95·9	93·7	94·7	92·9	94·7
Increments and standard errors	—	−0·05 ± 0·03	0 ± 0·04	−0·06 ± 0·03	−0·05 ± 0·06	—	—	—	—
(*c*) Concentration of solids in fat-free milk:									
Percentage	9·40	9·32	9·26	9·21	9·10	8·98	9·03	8·94	8·88
Relative content	100	99·1	98·5	98·0	96·8	95·5	96·1	95·1	94·5
Increments and standard errors	—	−0·08 ± 0·03	−0·06 ± 0·03	−0·05 ± 0·04	−0·11 ± 0·05	—	—	—	—

The lactation milk yields were corrected for length of "service period" by Sanders' corrections (1) and the results are shown in Table I and Chart 1. The increase in yield with age continues up to the fifth and possibly to the seventh lactation, after which the decline becomes increasingly rapid. It is convenient to express the increase in yield with age as a percentage of the first lactation yield. This has been done in Table I, and it will be seen that the fifth, sixth and seventh lactation milk yields are all more than 50 per cent. higher than the yield of heifers. The increments and their standard errors are shown in Table I for each lactation up to the fifth, after this age the number of animals available is too small to justify these figures. Most of the increments for milk yield are significant, but the figures for fat and solids-not-fat scarcely reach the standard usually accepted as proof of statistical significance. The smoothness of the curves on Chart 1(*b*) and (*c*), however, leave little doubt that the changes with age are not due to chance variations.

The general form of the curve of milk yield as affected by age is of the usual type, but the extent of the increases with age is greater than that reported by most other workers. The amount of the increase probably depends on the rate

of development of the animals and the management to which they are subjected. The writer has found that the increase in yield from the first lactation to the maximum was 10 per cent. in one herd, and 80 per cent. in another herd. Such variations appeared to be due to differences in management; in the first herd some of the records were made during the war period, and in the second a policy of high production was introduced during the time covered by the records. The management of the herd used for the present work remained, however, remarkably constant throughout, and it is believed that when a high standard of management is practised, the increase in yield associated with age is greater than the commonly accepted figure of about 30 per cent.

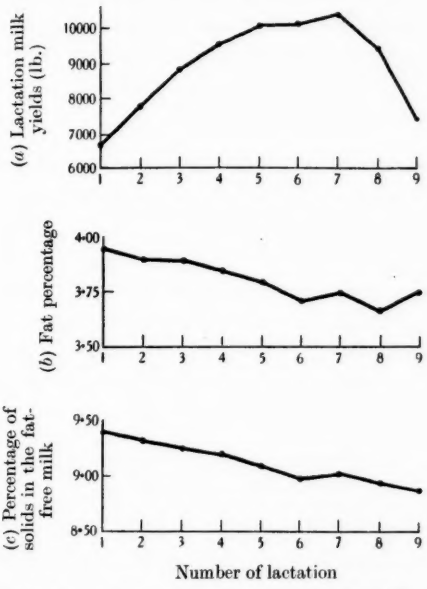

Chart 1. Variation with the age of the cow of (*a*) lactation milk yields, (*b*) the fat percentage of the milk, (*c*) the percentage of solids in the fat-free milk.

The concentration of fat and solids-not-fat shows a slight but almost regular decrease as age advances. The fat percentage declines from 3·95 in heifers to 3·74 for ninth calvers, a decrease of 0·21 per cent., or when expressed in relative figures, 100 and 94·7. The percentage of solids in the fat-free milk declines from 9·40 in heifers to 8·88 in the ninth lactation, a decrease of 0·52 per cent., or when expressed in relative figures, 100 and 94·5. Thus the reduction in the concentration of solids in the milk of older cows appears to apply to the fat and the solids-not-fat to a proportional extent.

The literature dealing with the influence of age on the fat content was reviewed by Mackintosh (2). Most workers obtained results similar to those on Chart 1(*b*), *i.e.* a slight decrease with advancing age.

The literature dealing with the effect of age on the solids-not-fat percentage of milk is scanty; the present work, however, suggests that the variations are somewhat similar for fat and solids-not-fat.

The reduction in the chemical quality of milk due to age commences too early to be attributed entirely to the onset of senility, and it is not unreasonable to assume that the decrease in quality between the first and second lactation is due to a cause other than that which is responsible for the decrease with age found in older cows. The secreting powers of first-calf heifers are characterised by exceptional persistence in yield as well as by an increase in the concentration of solids in the milk as the lactation progresses. Udder troubles are probably more common in older cows and these would tend to reduce the quality of milk produced. Pathological conditions of the udder, however, do not appear to be the only reasons for the gradual reduction in quality. The same tendency was found in cows which remained healthy and of normal secreting ability for long periods after the depression was observed.

Influence of preceding yield on milk secretion.

High yielding cows are almost always in negative calcium balance during the greater part of each lactation, and it was thought possible that this depletion of the mineral reserves of the body might be responsible for the lower concentration of solids in the milk of old cows. In order to test this hypothesis, all available lactations from second calvers or older cows were corrected for "service period" and then divided into three groups of approximately equal size. The groups were termed "high yields," "medium yields" and "low yields." The results are summarised in Table II.

Table II. *Changes in the concentration of the milk solids from one lactation to another.*

	Mean change in	
	Fat %	Solids in fat-free milk %
Lactations preceded by high yields	−0·041	−0·061
„ „ „ medium yields	−0·013	−0·035
„ „ „ low „	+0·042	−0·026

It will be observed that the concentration of solids in milk is slightly lower when the lactation is preceded by a "high yield" than when preceded by a "low yield." The differences are regular, but are very small, and a careful study of the figures suggested that the real reason for the change was not the size of the preceding lactation yield, but the cause noted under the next heading.

The influence of subnormal production on the composition of milk.

The classification of the records, which has just been described, indicated that when the lactation yield of a cow is below her normal secreting powers, the composition of the milk tends to be poorer during the subnormal lactation. To

test this further, all available lactation yields were corrected for "service period" by Sanders' corrections, and for age by convenient, but approximate, age correction factors. These factors assumed an increase in yield of 20 per cent. between the first and second lactations and 20 per cent. between the second and third lactations. No age corrections were applied to older cows and no lactation yields were used from cows after their eighth calf.

Each pair of consecutive lactation yields were then grouped according to whether the second lactation in the pair was:

(*a*) 30–49·9 per cent. lower than the first of the pair (40 per cent. decrease).
(*b*) 10–29·9 per cent. lower than the first of the pair (20 per cent. decrease).
(*c*) The difference did not exceed 9·9 per cent. (no change).
(*d*) 10–29·9 per cent. higher than the first of the pair (20 per cent. increase).
(*e*) 30–49·9 per cent. higher than the first of the pair (40 per cent. increase).

The results are shown in Table III.

Table III. *Changes in the composition of milk in successive lactations as affected by changes in the rate of secretion.*

	Proportional milk yield		Mean change in	
No. of pairs of lactations	Initial lactation	Subsequent lactation	Fat %	Solids in fat-free milk %
17	100	60	−0·09	−0·24
37	100	80	−0·06	−0·07
74	100	100	−0·03	−0·04
35	100	120	0	0
10	100	140	−0·01	−0·05

It will be observed that successive lactations in which the decrease in yields are greatest also show the greatest decrease in concentration of milk solids, particularly the solids-not-fat. When the yields remain fairly constant, or increase from one lactation to another, the concentration of solids tends to remain almost constant.

The changes in composition noted in Table II are probably due to the fact that high lactation yields are more likely to show a subsequent decrease than low lactation yields, while initial low yields are more likely to show a subsequent increase.

It is a widely accepted view that in a general population of cows, high milk yields tend to be associated with low fat percentage. This view is supported by the work of Gaines (3), and the present work need not be regarded as antagonistic. Whereas Gaines used a general population of cows, the present work deals with comparisons between successive lactation yields of the same cows. The new factor postulated here exerts an opposite but smaller influence than the ordinary reaction of quantity on quality of milk, and if both factors were operating in the data used by Gaines, he appears to have measured the difference between the two.

A probable explanation of this positive correlation between reductions in yields and solids content is suggested by the work of Davies (4) and Peskett (5). The general thesis of these workers is that one of the causes of low solids-not-fat

in milk is the infiltration of the body fluids into the milk and that this can be demonstrated in apparently normal milks. It appears possible, therefore, that the factors (possibly udder abnormalities) which depress the solids content of milk, also depress the rate of secretion. It would be premature to suggest that, when the normal concentration of the milk secreted by a cow is known, most decreases in the solids-not-fat content indicate pathological conditions of the udder. Such a conclusion could scarcely account for the difference between the solids content of the milk of barren cows and pregnant cows. Alternative reasons for the secretion of low solids, or even of body fluids not usually found in milk, might be the occurrence of fatigue in the udder tissue or the synthesising cells, or changes in the supply of hormones controlling milk secretion. Such changes could presumably occur in the absence of pathological changes in the udder.

SUMMARY.

1. The influence of the age of cows on the milk yield and on the milk solids is traced. The milk yield reaches a maximum at about the sixth lactation and then declines. The concentration of fat and solids-not-fat shows a gradual decline from the first lactation onwards.

2. During lactations in which the yield is below the normal productivity of a cow, the quality of the milk also tends to be subnormal.

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82. STUDIES IN THE RIPENING OF CHEDDAR CHEESE

IV. THE BACTERIAL FLORA OF CHEESE MADE FROM MILK OF A VERY LOW BACTERIAL COUNT

V. THE INFLUENCE OF THE RENNET FLORA ON THE FLORA OF THE CHEESE

VI. THE INFLUENCE OF THE STARTER ON THE RIPENING PROCESS

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In a previous communication (Allen (1)) it was shown that Cheddar cheese made from milk of a very low bacterial count (Grade A (T.T.)) exhibited a retarded rate of ripening, as evidenced by proteolysis and development of flavour and texture, when compared with similar cheese made from ordinary market milk, and the view was advanced that the miscellaneous flora of market milk produces sufficient proteolysis of the casein in the early stages of manufacture to provide the starter organisms with necessary nitrogen compounds for rapid growth.

In the present investigation there were three objects:

(1) To trace the development of the flora in cheese made from milk of a very low bacterial count, in order to be able tentatively to correlate any deficiencies in flavour and texture in the ripe cheese with deficiencies in flora during ripening.

(2) To find to what extent bacteria in the rennet contribute to the total flora of the cheese during ripening. Although the number from this source is initially insignificant it must be remembered that the flora of Cheddar cheese tends to develop in stages, one set of micro-organisms which have persisted through conditions adverse to their active multiplication taking the place of another set when the conditions have altered.

(3) To test the influence of the activity of the starter on the microbial flora and final degree of ripening of the cheese.

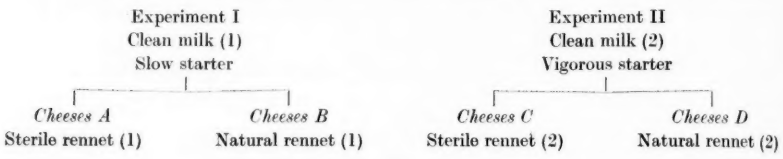
Milk of a very low bacterial count was also used in testing (2) and (3) in order to reduce to a minimum the competitive effect of other micro-organisms.

Experimental.

The cheeses were made in the laboratory from fresh Grade A (T.T.) milk in small tin vats of approximately 6 gallons capacity. Extraneous contamination was avoided by sterilising (by heat) the vats and all apparatus with which the

milk or cheese came into contact, and by covering the vats as far as possible during the making of the cheese.

Two separate experiments were run. In the first a slow starter and in the second a vigorous starter was used. In each experiment two vats were used and the milk divided between them—4 gallons in each. To one vat fresh natural rennet was added, and to the other the same rennet sterilised by filtration through a Berkefeld filter candle. The main differences in materials used for the four types of cheese may be represented briefly by the following scheme:



The slow starter was prepared by plating a commercial starter on dextrose-bean extract agar and selecting two strains of streptococci which when mixed produced 0·64 per cent. acidity in milk at 30° C. in 24 hours.

Four 1 lb. cheeses were made in each vat and a record of the process is given in Table I. To each vat 300 c.c. of starter were added while, for cheeses A and C, 8 c.c. of sterile rennet and, for cheeses B and D, 6 c.c. of natural rennet were used in each vat, tests with fresh milk having shown that the clotting activity of the rennet was reduced by filtration.

Table I. *Cheese-making record**.

	Exp. I. Slow starter		Exp. II. Vigorous starter	
	Cheeses A Sterile rennet	Cheeses B Natural rennet	Cheeses C Sterile rennet	Cheese D Natural rennet
Acidity of milk before starter added	0·15	0·15	0·17	0·17
Acidity of milk at renneting	0·21	0·21	0·22	0·22
Time taken to produce this acidity	4 hr. 0 min.	4 hr. 0 min.	2 hr. 30 min.	2hr. 48 min.
Rennet coagulation produced in	8 min. 55 sec.	5 min. 10 sec.	15 min.	6 min.
Time curd cut	5 hr. 33 min.	5 hr. 5 min.	3 hr. 30 min.	3 hr. 35 min.
Acidity of whey then	0·14	0·15	0·16	0·16
Time pitched	7 hr. 30 min.	6 hr. 55 min.	4 hr. 15 min.	4 hr. 28 min.
Time whey drawn	9 hr. 45 min.	9 hr. 5 min.	4 hr. 22 min.	4 hr. 47 min.
Acidity when whey drawn	0·19	0·19	0·19	0·20
Press whey acidity	0·54	0·68	—	—

* Times are reckoned from the moment of addition of starter.

It may be seen that, owing to the weak activity of the starter, acidity development in A and B was slow, and for this reason the curds were left to cheddar overnight before filling into the moulds and pressing. Small 1 lb. moulds were used and four cheeses made from each vat. After taking from the press each cheese was coated with sterile paraffin wax in order to prevent evaporation of moisture which would result in cracking of the cheese and growth of mould in the interior.

Bacteriological examination.

Five bacteriological examinations of each type of cheese were made over a ripening period of 6 months at intervals of 5 weeks. For each examination a whole cheese of each type was used, with the exception of the third examination, when a plug was removed for the purpose from each of the cheeses, with a sterile borer, the hole filled with sterile paraffin wax, and the cheeses replaced in the ripening room until used for the fourth examination.

The method adopted for sampling the cheeses was that given by Allen (*loc. cit.*). The 10 g. samples thus obtained were ground with sterile sand and dilutions prepared in saline at 45° C., with which to inoculate the following media:

Nutrient agar (*plates*). This was adjusted to *p*H 8·3 before sterilisation and encouraged the growth of organisms other than lactic acid bacteria—particularly micrococci.

Nutrient gelatin (*plates*). Used only to obtain counts of liquefiers.

Dextrose-bean agar (*plates*). Made from an extract of haricot beans and similar to the medium used by Maddock (2), except that dextrose was substituted for sucrose. It has been found particularly useful for the cultivation of lactic acid streptococci.

Marmite-whey agar at p*H* 4·2 (*shake cultures*). This medium contained 0·2 per cent. marmite and was used for selective cultivation of lactobacilli, being buffered to the required *p*H by addition of citric acid and potassium citrate as directed by Davis (3). At first a similar medium containing no marmite was used, but comparison with the marmite medium showed that the latter encouraged a much quicker growth of lactobacilli and resulted in much larger colonies, though the final count was not appreciably different.

Beerwort agar at p*H* 3·5 (*plates*). The *p*H was obtained by the method of Davis (*loc. cit.*), the object of the medium being to isolate yeasts. It may be seen that no yeasts were found in the dilutions tested but that on the other hand many of the lactobacilli flourished on this medium in spite of fully aerobic conditions.

Litmus milk and marmite-litmus milk. At the fourth examination these media were used to obtain approximate counts of organisms in the cheeses by the dilution method, with a view to comparing their value with the other media being used. They were also used for cultivating lactic acid bacteria isolated from single colonies on other media. The marmite-litmus milk contained 0·2 per cent. of marmite.

Casein digest agar. This consisted of a peptic digest of casein to which were added mineral salts, dextrose and agar, and was used at the fifth examination for preparing shake cultures similar to those described for marmite whey agar, with the object of comparing the value of this medium with the others used.

Sugar broths. All sugar broths used for testing the reactions of isolated cultures contained 0·2 per cent. marmite, since many of the lactic acid bacteria fail to ferment sugars in the absence of a suitable growth stimulant.

Nature of micro-flora.

The counts obtained at intervals during the ripening of the four types of cheese are shown in Table II. In general it may be seen that for all cheeses by far the highest counts were found on dextrose-bean agar—due to the fact that this medium allowed good growth of starter organisms. In Exp. I these

Table II. *Bacterial counts of cheeses (millions per gram) on various media at intervals during ripening.*

Exp. I.

Period of ripening (weeks)	Gelatin liquefiers		Nutrient agar		Dextrose bean agar		Whey agar *p*H 4·2		Beerwort agar *p*H 3·5	
	A	B	A	B	A	B	A	B	A	B
5	0·64	0·6	0·891	0·523	159	104	1·2	1·9	0·259	0·084
10	0·015	0·036	0·178	0·0102	60·0	69·0	0·24	0·73	0·148	0·273
15	0·11	0·006	0·218	0·0005	14·2	12·0	0·57	0·49	0·247	0·073
20	0·001	—	0·069	0·0002	28·0	12·8	1·15	0·83	0·368	0·395
26	0·001	0·00015	0·00134	0·00016	7·6	8·52	0·32	0·009	0·14	0·255

	Litmus milk		Marmite litmus milk		Casein digest agar					
20	10	1	1	10	—	—	—	—	—	—
26	—	—	—	—	3	6				

	Nutrient agar	Casein agar	Dextrose bean agar	
Bacteria per c.c. of fresh milk	9,500	—	6,400	*B. Coli* absent from 1 c.c.
Bacteria per c.c. of rennet	56,000	25,000	—	

Exp. II.

Period of ripening (weeks)	Gelatin liquefiers		Nutrient agar		Dextrose bean agar		Whey agar *p*H 4·2*		Beerwort agar *p*H 3·5*	
	C	D	C	D	C	D	C	D	C	D
4	0·0585	0·0485	0·055	0·0333	5·0	9·8	—	—	—	—
9	0·054	0·070	0·092	0·155	0·460	1·24	—	—	—	—
14	0·012	0·013	0·0112	0·0095	0·42	0·61	—	—	—	—
19	0·0001	0·002	0·0013	0·0059	1·07	0·124	—	—	—	—
25	0·003	0·0001	0·0004	0·0012	0·237	0·009	—	—	—	—

	Litmus milk		Marmite litmus milk		Casein digest agar					
19	0·1	0·1	0·1	0·1	—	—	—	—	—	—
25	—	—	—	—	0·44	0·012				

	Nutrient agar	Dextrose bean agar	Litmus lactose agar	
Bacteria per c.c. of fresh milk	9,000	8,600	—	*B. Coli* absent from 1 c.c.
Bacteria per c.c. of rennet	9,400	3,200	10,500	

* On these media no colonies were observed in the lowest dilution used, *i.e.* 1/100 c.c.

organisms, at the time of the first examination and subsequently, were present in much greater numbers than in Exp. II. Since in the latter experiment a more vigorous starter was used it may perhaps be assumed that the organisms had attained maximum growth more rapidly in the very early stages of ripening and had declined considerably by the time of the first examination.

The flora on nutrient agar (apart from pin-points) was found to consist, at all stages of the investigation, almost entirely of micrococci giving rise to yellow colonies. The majority of these liquefied gelatin, were strongly haemolytic and proved to be cultures of *Staphylococcus aureus*, an organism present in fairly large numbers in the early stages and which may have come from the udder. Three cultures of micrococci (of a total of seventy-one examined) gave a canary yellow growth on nutrient agar, produced very slow peptonisation in litmus milk, very slow liquefaction of gelatin and no action in carbohydrates. They corresponded closely with the description of *Micrococcus mucofaciens* Thöni and Thaysen. Examination of the gelatin plates showed that micrococci were responsible for the counts of liquefiers.

The colonies on dextrose-bean agar proved in the first four examinations to be mainly streptococci, with much smaller numbers of staphylococci. The greater vigour of the starter used in Exp. II was reflected in the early stages of ripening by the action of the organisms in litmus milk, a greater proportion of acid coagulating types being obtained from these cheeses than those in Exp. I.

The effect of yeast extract (in the form of marmite) on the growth of the lactic acid streptococci isolated from this medium was very marked. Thus the majority produced no change in litmus milk after incubation at 30° C. for several days. Thirty-nine cultures, obtained from the four varieties of cheese at different periods of ripening, were tested by adding sterile marmite solution to make the final concentration 0·2 per cent. and re-incubating. It was found that thirty-five showed an acid coagulation, usually in 2–3 days—only four cultures still showing no change.

Many of these organisms showed a further peculiarity in litmus milk. Even when yeast extract was provided and good growth and coagulation had taken place the curd was usually not the typical homogeneous curd with reduction of the litmus. It was generally a uniform pink and in many cases was very spongy as though broken up by the evolution of gas. Thus of seventy-six cultures tested in marmite-litmus milk, eight (or 10·3 per cent.) produced a typical lactic acid curd, twenty-four (or 30·8 per cent.) produced a uniform red curd, and twenty-eight (or 36·8 per cent.) produced a spongy curd with expressed whey.

The characters of twenty-seven strains isolated from dextrose-bean agar were tested in litmus milk, marmite-litmus milk, gelatin, nitrate broth and in various sugar broths. None liquefied gelatin nor reduced nitrates. They showed a fairly wide range of carbohydrate fermentations, but this criterion alone is unsatisfactory for distinguishing lactic acid bacteria. On the other hand, those organisms producing no change in litmus milk after several days' incubation and a characteristic spongy acid curd after addition of yeast extract, appeared to be differentiated from the rest, and moreover formed nearly 40 per cent. of the total flora isolated from this medium. In general these strains nearly always fermented dextrose, lactose, sucrose, maltose, and sometimes raffinose and salicin, but did not ferment mannite and inulin. The sponginess of the curd seemed to indicate gas formation in the milk, and careful daily observation of

inoculated sugar broths revealed small but definite quantities of gas in several of the broths, the amount varying with the nature of the sugar.

It is of interest to note here that Evans (4) recorded the presence in Cheddar cheese of three types of *Streptococcus* other than those of the *S. lactis* type. One of these was an organism producing only a slight acidity in milk, but forming a gassy curd in peptone milk and fermenting lactose and sucrose, sometimes raffinose and salicin, but not fermenting mannite and glycerol. Evans identified this organism as *S. kefir*, and its characters are very similar to those of the strains described here. On the other hand it must be borne in mind that some bacteria which do not ordinarily form gas in carbohydrates may be induced to do so under certain conditions, and it seems possible that the organisms noted here were derived from more typical strains of lactic acid streptococci whose characters had become modified in the course of cheese ripening. In fact it seems unlikely that these bacteria would otherwise occur in large numbers in cheese made from very clean milk, sterile rennet and a starter of two selected

Table III. *Nature of flora isolated from dextrose-bean agar after 25–26 weeks' ripening.*

Source	Cultures tested	Morphology	Reaction in marmite-litmus milk
A	12	(8) Rod	Homogeneous acid clot. Litmus reduced
		(3) Coccus in pairs	Spongy acid clot
		(1) Varying from coccus to rod	Spongy acid clot
B	12	(9) Rod	Homogeneous acid clot. Litmus reduced
		(3) Coccus in pairs	Spongy acid clot
C	10	(8) Rod	No change
		(1) Coccus mainly in pairs	Acid clot with expressed whey
		(1) Coccus in pairs and tetrads	No change
D	5	(4) Rod	No change (only 1 tested)
		(1) *Staphylococcus*	

pure strains. Stained preparations showed pleomorphism, some of the cultures being mainly oval cells in pairs, short chains or clusters, and others (particularly those isolated in the later stage of ripening) showing in the same strain cells varying from oval to short granular rods.

In the final examination of the cheeses (*i.e.* after 25–26 weeks' ripening), thirty-nine cultures were isolated from the dextrose-bean agar plates from the four types of cheese, and their morphology and reactions in marmite-litmus milk are shown in Table III. Thus in both cheeses in Exp. I the majority of organisms were lactobacilli giving the typical acid-reduced clot in marmite-litmus milk. The remaining cultures were the peculiar cocci referred to above which gave a spongy acid clot in the same medium.

In the case of the cheeses in Exp. II the flora from both C and D consisted mainly of Gram-positive rods producing no change in marmite-litmus milk, but the counts in any case were comparatively small.

Tests with eight strains of the lactobacilli from A and B showed that all fermented dextrose, lactose, maltose, mannite and salicin, and none fermented inulin, but they varied in their action on sucrose and raffinose.

Table II shows that at each stage of ripening investigated appreciable counts were obtained in whey agar at *p*H 4·2 for the cheeses A and B. All the organisms so isolated were found to be strains of lactobacilli, and it is noteworthy that whereas aciduric organisms of this type are usually associated with the later stages of cheese ripening, in this investigation they were found in the highest numbers in this medium at the first examination, and showed a marked tendency to decrease in numbers in the later stages. The strains isolated were divisible into two types on the basis of their reaction in litmus milk at 37° C.: (1) those which produced a firm acid clot with reduction of the litmus, after several days' incubation, (2) those which, after incubation for 16 days, had produced comparatively little acidity, varying from that sufficient to form a very soft coagulum to that detectable only by a slight colour change in the litmus. Addition of marmite to the latter induced a typical acid clot with reduction of the litmus in 3–7 days. Of forty strains tested in this way only four were of type (1), the remainder being of type (2). Ten strains varying in vigour of growth and acid production, and isolated from both types of cheese at different periods of ripening, were tested for carbohydrate fermentations. All strains fermented dextrose, lactose, sucrose, maltose, mannite and salicin with production of acid but no gas, and none of the strains fermented raffinose nor inulin.

No lactobacilli were isolated from cheeses C and D in any of the media.

On beerwort agar at *p*H 3·5 appreciable counts were obtained for the cheeses A and B but none for C and D. After 14–16 days' incubation at 30° C. surface colonies were quite large, but examination showed them to be lactobacilli of the same type as those isolated from the whey agar shake cultures, varying in their vigour of growth in litmus milk and markedly stimulated in this respect by addition of yeast extract.

From the counts obtained in litmus milk and marmite-litmus milk it is obvious that the latter offer no advantages over the other media used. Stained preparations showed that at this stage (19–20 weeks) streptococci in pairs and short chains were predominant.

The counts in casein agar shake cultures at the final examination (25–26 weeks) were comparable with those on the dextrose-bean agar plates. The types isolated from this medium were a mixture of streptococci and lactobacilli (cheeses A and B only) with varying reactions in litmus milk.

Chemical examination.

At the fifth and final examination of the cheeses (25–26 weeks old) chemical tests were made in order to see whether any outstanding differences in proteolysis were revealed. For this purpose the portion of each cheese remaining from the bacteriological examination was grated and sampled and about 5 g. accurately weighed. This was ground with sand, mixed with 30 c.c. water at 55° C. and titrated with standard sodium hydroxide to the first end-point, using phenolphthalein as indicator. A neutralised mixture of absolute alcohol

(100 c.c.), 40 per cent. formalin (5 c.c.) and water (10 c.c.) was then added and the titration continued to the second end-point. The first titration was then taken as a measure of the acidity of the cheese and the difference between the first and second readings as a measure of peptide scission.

At the same time the nitrogen content of each cheese was determined by Kjeldahl's method. All determinations were made in duplicate and results for acidity and alcohol-formalin titration calculated per gram of nitrogen. Table IV shows the figures obtained. It may be seen that there is no significant difference in extent of proteolysis between the cheese made from sterile rennet and that made from ordinary rennet, either in Exp. I or in Exp. II. On the other hand both cheeses in Exp. II show an alcohol-formalin titration approximately twice that of the cheeses in Exp. I.

Table IV. *Acidity, alcohol-formalin titrations and nitrogen contents after 25–26 weeks' ripening.*

	Cheese			
	Exp. I		Exp. II	
	Sterile rennet	Ordinary rennet	Sterile rennet	Ordinary rennet
Acidity (c.c. *N*/10 NaOH per g.)	(1) 2·91 (2) 2·80 Av. 2·86	(1) 3·54 (2) 3·36 Av. 3·45	(1) 2·88 (2) 2·94 Av. 2·91	(1) 2·65 (2) 2·74 Av. 2·70
Alcohol-formalin titration (c.c. *N*/10 NaOH per g.)	(1) 2·20 (2) 2·36 Av. 2·28	(1) 2·08 (2) 2·17 Av. 2·13	(1) 3·97 (2) 3·94 Av. 3·96	(1) 4·03 (2) 4·08 Av. 4·06
Nitrogen content (%)	(1) 3·85 (2) 3·83 Av. 3·84	(1) 3·78 (2) 4·16 Av. 3·97	(1) 3·65 (2) 3·66 Av. 3·66	(1) 3·55 (2) 3·27 Av. 3·41
Alcohol-formalin titration calculated per g. N	56·77	53·65	108·20	116·13
Acidity (c.c. *N*/10 NaOH) calculated per g. N	74·48	86·90	79·51	79·18

Flavour and texture of the cheeses.

There were no apparent differences in flavour and texture between the cheeses made from sterile rennet and those made from ordinary rennet in either of the experiments. The cheeses in Exp. I had a very mild flavour and rather soapy texture even after 26 weeks' ripening. On the other hand those in Exp. II showed a distinctly sharp flavour and more open texture fairly early in the process of ripening, and a slight bitterness which was most distinct at the fourth examination (19 weeks old). After 25 weeks' ripening these cheeses still showed a noticeably sharper and fuller flavour than those in Exp. I, and there was a slight but definite bitterness.

Discussion.

The bacteria in Cheddar cheese may be grouped as follows: (1) starter organisms, (2) the rennet flora, (3) the udder flora, (4) adventitious bacteria arising through contamination of the milk or, to a less extent, of the cheese during manufacture. The experiments recorded here may be regarded as testing the extent to which bacteria in the first three groups contribute to the

ripening. Since milk of a very low bacterial count was used and subsequent contamination largely avoided they may also be claimed to test to some extent the effect of a deficiency of bacteria in group (4).

It is evident that the rennet bacteria did not make a significant contribution to the total flora of the cheeses, whether made with a weak or with a vigorous starter, nor was any influence in this respect revealed by a chemical examination of the ripe cheeses, nor by their flavour and texture.

The normal udder flora consists almost exclusively of micrococci and staphylococci, and in these experiments *Staphylococcus aureus* was the only organism of this type which was found in appreciable numbers. The fact that the count was considerably higher in the cheeses made with a weak starter than in those made with a vigorous starter seems to indicate the influence of the latter in checking the growth of this organism.

In Exp. II, apart from a vigorous growth of starter organisms very early in ripening, the cheeses failed to show significant counts of micro-organisms of any type and no lactobacilli were detected at any stage. In Exp. I, on the other

Table V. p*H values of cheeses at intervals during ripening.*

Exp. I			Exp. II		
Period of ripening (weeks)	Sterile rennet	Ordinary rennet	Period of ripening (weeks)	Sterile rennet	Ordinary rennet
5	5·10	5·03	4	4·97	5·11
10	5·15	5·20	9	5·15	5·10
20	5·22	4·98	19	5·32	5·22
26	5·46	5·11	25	5·16	5·22

hand, the lactic acid streptococci, although predominant for the first 20 weeks of ripening, were replaced later by lactobacilli which attained several millions per gram. Nevertheless, the former cheeses exhibited much more proteolysis and a fuller flavour than the latter. It follows from this that a vigorous starter contributes materially towards the ripening process and is much more effective in this respect than a slow starter. That the effect was not due to greater acid production is shown by Table V, from which it may be seen that the *p*H of all types of cheese were approximately the same.

We must therefore conclude that the symbiotic effect of a mixture of starter organisms induced greater proteolysis than the action of two species. This confirms the observations of Barthel and Sadler (5) that the amount of amino nitrogen produced by a single member of the *S. lactis* group was much less than that produced by a mixture of strains.

Lactobacilli were isolated only from cheeses in Exp. I. Those appearing in the acid media showed a general tendency to decrease as ripening progressed, while those found on approximately neutral dextrose-bean agar after 25–26 weeks' ripening formed 65–75 per cent. of the flora, numbering 5–6 millions per gram of cheese, and were predominant. Those isolated from the two acid media were also more uniform in character than those from dextrose-bean

agar. It appears, therefore, that an appreciable proportion of lactobacilli were inhibited by the acid media.

The absence of lactobacilli in the cheeses showing greater proteolysis and fuller flavour and their presence in considerable numbers in cheeses showing comparatively little proteolysis and a lack of flavour is striking. The work of Hastings, Evans and Hart (6), Harrison (7), Harrison and Connell (8), Evans, Hastings and Hart (9) and Kulp and Rettger (10) has shown that lactobacilli are normally predominant in the later stages of Cheddar cheese ripening and that they contribute materially to proteolysis and flavour production. In our own experiments, on the other hand, the effect of the lactobacilli in these respects was evidently small compared with that of the streptococci.

This does not necessarily mean that appropriate species of lactobacilli may not have an effect when the conditions are suitable. It must be remembered that the source of lactobacilli in cheese is the milk and that the chief mode of entry into the milk is *via* the milking utensils. Under proper methods of clean milk production the latter are sterilised by steam and any lactobacilli destroyed. That the effect may be to eliminate this genus of organisms from the milk entirely is shown by the fact that the cheeses made from one lot of milk (Exp. II) were completely deficient in them. Since the milk used in Exp. I was of the same grade we may assume that initial contamination by lactobacilli was small and that the number of species was restricted. Thus those present may not have been actively proteolytic, and in any case the effect of a very few species would probably be much less than the symbiotic effect of a larger number.

It is significant that nutritional factors had a very marked influence on the growth and acid formation of the lactic acid bacteria which were isolated—both streptococci and lactobacilli. That these organisms are sensitive in their nitrogen requirements was shown by Orla-Jensen (11), but the importance of this in cheese ripening has not, perhaps, been fully realised. The majority of lactic acid bacteria isolated here required a suitable source of nitrogen (it may be assumed that this is what the marmite actually provided) before they would grow actively in milk. *It follows that these bacteria will require a similar source of nitrogen before they attain vigorous growth in the cheese.* As suggested previously (Allen, *loc. cit.*) this nutritional factor is probably supplied in market milk cheese by the decomposition products of proteolytic bacteria originally present in the milk, whereas in cheese made from a high grade of milk it is absent.

Although the cheeses in Exp. II showed greater proteolysis and a fuller flavour than those in Exp. I their flavour was certainly not nearly so full and pungent as is desirable, and there was in addition a distinct bitterness. This suggests that, although the symbiotic effect of the streptococci, combined with the action of enzymes, had broken down the protein to an appreciable extent, the necessary mechanism to complete the process was lacking. It is probable that the missing factor was the correct type of lactobacilli growing in the presence of an adequate source of nitrogen.

We may conclude tentatively from these experiments, therefore, that at least three factors are essential to induce proper ripening in cheese made from milk of a low bacterial count:

(1) A vigorous starter consisting of a mixture of species of lactic acid streptococci.

(2) Lactobacilli of a type able to carry still further the protein decomposition initiated by enzymes and lactic acid streptococci. Since these are likely to be absent from the milk when produced they must be added in the form of cultures.

(3) An adequate source of nitrogen to allow full growth for the starter organisms and lactobacilli.

It is hoped to carry out further experiments to discover the best methods of ensuring these factors.

Conclusions.

1. The flora of the rennet could not be detected as contributing towards the micro-organisms concerned in the ripening of cheeses made either with a vigorous or with a slow starter.

2. In cheeses made from milk of a very low bacterial count the predominant flora consisted, for the first 19–20 weeks at least, of lactic acid streptococci. Where lactobacilli were present initially these organisms displaced the streptococci at later stages of ripening and became predominant.

3. The effect of a vigorous starter in inducing subsequent proteolysis is very marked by comparison with that of a weak starter consisting of two pure species of streptococci. This is probably due to the symbiotic effect of a mixture of organisms.

4. In milk produced under strict conditions of cleanliness either lactobacilli may be entirely absent or those few species which are present may not be of a type able to contribute towards the ripening process. One factor, usually regarded as essential to produce full flavour and proteolysis in Cheddar cheese, is therefore missing.

5. The majority of lactic acid streptococci and lactobacilli isolated throughout the ripening process were of a type unable to grow vigorously in milk without the addition of a suitable source of nitrogen. This nutritional factor is missing in milk of a low bacterial count and therefore, in order to ensure proper growth of these organisms in cheese made from such milk, it must be provided artificially.

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83. OBSERVATIONS ON SUDDEN CHANGES IN THE RATE OF ACID FORMATION IN MILK BY CULTURES OF LACTIC STREPTOCOCCI

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It is a curious fact that although cultures of lactic streptococci have been used as starters in the manufacture of cheese during the past thirty years, there are no published accounts (so far as we are aware) of attempts to explain the dramatic changes in activity which such cultures exhibit from time to time even when they are subcultured daily under apparently constant conditions. A large mass of work has been published dealing with the influence of the lactic streptococci on the ripening of and development of flavour in cheese, but the very important part played by the bacteria in the formation of acid during the process of manufacture has either not attracted the attention of research workers or has proved a subject of exceptional difficulty. In the manufacture of many types of cheese, particularly those of soft texture, the demands for acid production made upon the starter are not very heavy and consequently slight variations of activity do not obtrude themselves unduly on the attention of the cheese maker. With Cheddar cheese, however, the case is different. The particles of curd in which the starter is expected to develop are relatively dry; and the temperature to which the curd is raised (normally 100° F., in extreme cases 105° F.) in order to promote the expulsion of moisture is often high enough to retard quite markedly the growth of the bacteria. Thus in the manufacture of Cheddar cheese it is necessary that the starter should produce, within six hours of the start of the process, the equivalent of 1 per cent. of lactic acid in the whey under adverse conditions both of moisture supply and temperature. It is not to be wondered at, therefore, that starters frequently fail to attain this performance. Apart, however, from the cases where the starter is rather slow in producing the required amount of acid there are those instances where a starter, having functioned satisfactorily for some time, suddenly undergoes such a sudden loss in vitality that it fails completely to approach the required standard of performance. In the large Cheddar cheese factories in New Zealand failure of starter cultures even for a single day is a serious matter from the economic point of view; therefore attention has been focused for some time at this Institute on the problem presented by the apparently uncontrollable variations in starter activity. The present paper

deals with some cases of sudden failure in growth of certain cultures of lactic streptococci where the cause was traced down to variations in the respiratory processes of the bacteria which rendered them particularly sensitive to slight alteration of the conditions existing in the milk medium.

It has been the practice for some years in this laboratory to check the activity of starter cultures which are to be used in the making of experimental cheeses. The "vitality test" described by Whitehead and Cox(1) is used for this purpose; it is designed to simulate on a small scale the process which is followed in the cheese vat. Some years ago it was noticed on one or two occasions that although the starter which was in use gave normal results in the vitality test, it failed almost entirely to produce acid in the cheese vat. The culture tested in the laboratory and that used in the experimental factory had both been sown from the previous day's mother culture, the only difference being that the former was a small quantity prepared in a conical flask while the latter was prepared in several gallons of milk in a tinned steel can in the factory. When the phenomenon was observed for the third time in April 1931 it was submitted to a detailed investigation. It was found that whereas the mother culture, prepared daily in the laboratory in 200 c.c. of separated milk in a conical flask plugged with cotton-wool, remained relatively constant in its activity from day to day, a larger bulk of culture prepared in a factory can and sown from the laboratory culture was liable to lose its activity with dramatic suddenness. It was all the more extraordinary that a culture from the can which proved quite inactive in the vat or in a vitality test was quite normal in appearance. The milk was clotted and had an acidity of 0·8–0·9 per cent. lactic acid; the organisms in the culture were normal in appearance under the microscope. When, however, a further batch of milk was inoculated from the inactive culture and incubated at 20° C. in the usual way it failed to clot in 24 hours, and usually clotting did not take place until after a lapse of 3 days.

In an enquiry into the cause of the phenomenon the first possibility considered was that contact with the metal of the can during the pasteurising process affected the milk as a medium. It was readily shown, however, that the phenomenon occurred equally well in vessels of tinned steel, enamelled iron, stainless steel, and glass. An experiment such as the following showed that the slight difference in the method of application of heat to the milk in the factory as compared with the method used in the laboratory was without significant effect, and that the shape of the vessel used was apparently the crucial factor. A metal can containing 3 gallons of milk and a flat-bottomed round glass flask containing 1 gallon of the same batch of milk were immersed in the water bath at the factory and the milk was pasteurised by admitting steam to the water until the temperature was raised to about 200° F.; this temperature was maintained for 1 hour. Cold water was then run through the water bath until both lots of milk were cooled to 70° F. Both vessels were inoculated from the same sample of mother culture from the laboratory and

allowed to stand at 68–70° F. overnight. The next morning both cultures had a similar appearance and had developed approximately the same acidity (0·8–0·9 per cent. lactic acid). The two starters were added in similar amounts to two small vats of pasteurised milk taken from the bulk supply. During the process of cheese manufacture the culture from the glass flask proved quite active, while the culture from the can was absolutely inactive. Vitality tests on the two cultures in the laboratory confirmed the result. Several successive experiments bore out this finding, and in these it was also shown that milk pasteurised and cooled in a glass beaker (*i.e.* a vessel similar in shape to the can) holding about 1 gallon also gave rise usually to an inactive starter. Since it had now become apparent that vitality tests in the laboratory gave a reliable indication of how starters would behave in the vat (at least in so far as a differentiation between active and "dead" starter cultures was concerned) investigation of the phenomenon was continued only in the laboratory. The general plan of the experiments, which were performed almost daily, was as follows. Six glass vessels (beakers or flasks), each containing 200 c.c. of milk, all from the same bulk supply, were immersed in a bath of water which was raised gradually to boiling-point. Boiling was continued for half an hour and then the samples were cooled to a temperature of 20° C. They were all inoculated with an equal number of drops of the starter culture under investigation and incubated at 20° C. for about 20 hours. The acidity of the clotted cultures was determined and was always approximately the same for all. Vitality tests were conducted in six vessels filled from the one batch of milk and with the addition of an equal amount (1 per cent.) of the six cultures. The following figures are taken from the results of a typical experiment in which the milk for the cultures was pasteurised in beakers.

Table I. *Acidities as parts per thousand lactic acid.*

	Culture No.						Mother culture from conical flask
	1	2	3	4	5	6	
First reading	1·7	3·1	3·4	1·7	1·7	3·0	3·1
Second reading	2·4	6·2	6·6	2·6	2·3	6·2	6·0
Increase	0·7	3·1	3·2	0·9	0·6	3·2	2·9

It will be observed that whereas three of the cultures gave normal acidity increases in the whey draining from the curd and were equal in activity to the mother culture, the other three cultures gave only very low acidity increases. The relative numbers of active and inactive cultures varied in each experiment. On occasions the phenomenon seemed to be in abeyance for a period of several days and all the cultures were quite active; but for a long period at least one out of the six proved to be "dead" as judged by the results of the vitality test. As had been found with "dead" cultures in cans in the factory, a further subculture from an inactive culture was very slow in clotting milk. Many variations in the conditions of pasteurisation of the six samples were tested

out. The phenomenon occurred in both whole and separated milk; the temperature of pasteurisation could be varied over a wide range with no difference in result; the amount of mother culture inoculum used did not appear to be significant. The only factor which induced a marked difference appeared to be connected with the shape of the vessels in which the milk was pasteurised. No inactive cultures were obtained when conical flasks were used instead of beakers. This is evidenced in the results of a typical experiment.

Table II. *Acidities as parts per thousand lactic acid.*

	Cultures prepared in beakers						Cultures prepared in flasks						Mother culture
	1	2	3	4	5	6	1	2	3	4	5	6	
First reading	1·6	2·1	2·7	2·6	1·7	1·5	2·6	2·7	3·1	3·0	2·6	3·2	2·5
Second reading	2·1	3·8	5·6	5·0	2·1	2·1	5·6	5·7	6·9	6·0	5·7	6·6	5·6
Increase	0·5	1·7	2·9	2·4	0·4	0·6	3·0	3·0	3·8	3·0	3·1	3·4	3·1

These results incidentally provided an explanation for the fact that throughout the series of experiments the mother culture, maintained in a conical flask, never became inactive.

The starter was so erratic from time to time in its response to the experimental conditions that consecutive work on the problem was very difficult, and no progress towards an elucidation of the phenomenon was made until on one occasion an active and an inactive culture from the same series of beaker cultures were streaked on the surface of yeast-whey agar. After incubation of the plates, an examination of the growth under the low-power lens of the microscope showed that there were two types of colony in the cultures. The growth from the active culture consisted of a mixture comprising about 75 per cent. of smooth colonies and 25 per cent. of rough or granular colonies, while the growth from the inactive culture consisted in the main of the rough streptococcal colonies. Specimens of the two types of colony were picked off and grown in sterilised milk. The rough types proved to be slow clotters, taking two or three days at 20° C. to coagulate milk; the smooth types coagulated milk overnight at 20° C.

The experiments in which six beakers of milk were pasteurised and inoculated with starter culture were now repeated; but in place of the mixed culture a pure smooth strain culture was used. The essential phenomenon was found to occur but in an apparently exaggerated form. Some of the beaker cultures coagulated normally on incubation overnight, but a certain proportion (varying in each experiment) did not even coagulate and showed only a very feeble growth of the smooth strain streptococci. The factor or factors which conditioned the erratic behaviour of the smooth strain cultures seemed to be identical with those which affected the whole mixed starter culture. The only difference was that whereas the cultures prepared in beakers from the mixed starter all invariably clotted and appeared normal until carried through to the next generation, the cultures in beakers from the single strain demonstrated their sensitivity, if at all, in the first generation by failing to clot

the milk in 24 hours. It is as well to state at this point that the property of smoothness in the colonies of the sensitive *Streptococcus* proved to have no demonstrable connection with the phenomenon; a change from insensitivity to sensitivity is not accompanied by a change in the nature of the colony, nor are all sensitive streptococci smooth strains.

After many trials a first step towards the solution of the problem was made by the discovery that the single-strain cultures were sensitive to aeration of the milk into which they were inoculated. This was definitely shown to be the main if not the only factor which determined their erratic behaviour. Milk which was pasteurised, cooled, and then aerated by pouring from vessel to vessel would not permit rapid growth of the organisms. The condition existing in the beaker cultures was evidently a borderline one. In some beakers there was during the cooling period a reabsorption of atmospheric oxygen sufficient to prevent ready growth of the organisms; in conical flasks there was rarely sufficient reabsorption, but even where flasks were used it appeared necessary to insert a plug of cotton-wool in the neck to make absolutely certain that conditions in the milk medium were correct for rapid growth of the organisms. No matter what the type of vessel used, any aeration after the pasteurising process produced an inhibitory condition in the milk. It is of course well known that the lactic streptococci show a preference for anaerobic conditions, but these experiments are the first indication, so far as we are aware, that on occasion a *Streptococcus* is so extremely sensitive to its environment that its growth is very markedly delayed when the liquid medium in which it is growing is aerated. It must be emphasised that the inhibition observed in aerated milk was not absolute or permanent; the single-strain cultures would eventually coagulate aerated milk but only after incubation for several days. The most remarkable feature of the phenomenon was the extremely narrow borderline, as it were, which separated suitable from unsuitable respiratory conditions for the organisms.

The smooth-strain cultures exhibited sensitivity for a period of about 3 weeks when suddenly they became entirely insensitive to aeration of the culture milk. They were subcultured daily and tested from time to time but appeared absolutely normal for a period of 9 months. The mixed starter culture from which they had been isolated simultaneously ceased to exhibit the phenomenon and consequently work on the problem was held up.

In September 1933 the phenomenon recurred suddenly. A pure culture of a strain of *Streptococcus cremoris* had been in use for several weeks as a starter in the experimental factory. It had produced acid normally throughout the process of cheese manufacture. Suddenly on one day it was found that the culture in the starter can was not even coagulated, although the mother culture maintained in a conical flask was clotted as usual. Investigation in the laboratory showed that the culture had become sensitive to aeration of the milk. Furthermore, three other pure strains of streptococci (two of them were the strains isolated 9 months previously) had likewise become sensitive.

Work was concentrated on the pure strain (HYW) which had been in use as a starter in the factory. The previous findings with sensitive cultures were confirmed. It was further shown that agitation of pasteurised milk *in vacuo* did not render it inhibitory towards the organisms; therefore the absorption of gas (almost certainly oxygen) is the important factor connected with aeration. Evidence was also obtained that the oxygen dissolving in the milk during aeration entered into chemical combination with some constituent, since passage of hydrogen through aerated pasteurised milk never removed its inhibitory properties. From this point of view the phenomenon seems akin to that described by Wright (2) who found that the growth of the *Pneumococcus* is adversely affected when peptone is present in the medium in the oxidised state. The only physical treatment in our experiments which rendered aerated milk once more suitable for ready growth of the sensitive streptococci was a further subjection to pasteurisation and an avoidance of subsequent aeration.

Mechanism of the inhibition of streptococcal growth produced by aeration of milk.

In a consideration of the possible mechanism of the reaction described above the authors thought along two lines. A good deal of work in recent years has tended to show that bacteria in the initial stages of growth are often sensitive to the level of oxidation-reduction potential in media in which they are sown. The possibility that aeration acted by influencing the oxidation-reduction potential of the milk had to be considered. Secondly, the work of M'Leod and Gordon (3, 4), and Avery and Morgan (5) has emphasised the importance of peroxide formation in the presence of oxygen, which in the case of organisms without catalase may lead to inhibition or death of the organisms. The streptococci do not form catalase but they are not so invariably peroxide producers as the pneumococci, nor are they so extremely sensitive to its action as the anaerobic bacteria. Still it is possible that an enhanced power of producing peroxide in the presence of atmospheric oxygen or, on the other hand, an unusual sensitivity to the action of peroxide, might provide an explanation for the abnormal behaviour of these peculiar lactic streptococci. These two possible explanations are not mutually exclusive of one another. Inhibition due to peroxide may be the mechanism of a reaction which can be expressed in terms of oxidation-reduction potential. A definite level of oxidation-reduction potential may be necessary before peroxide can be formed.

Search for evidence of inhibition by peroxide.

Graduated amounts of hydrogen peroxide were added to several series of flasks of steamed separated milk. The different series were inoculated with *Str.* HYW and with cultures of normal streptococci, and incubated at 20° C. overnight. It was found that there was no significant difference in the con-

centrations of peroxide necessary to inhibit the growth of sensitive and normal streptococci. Both types clotted milk containing up to 12 parts per million of peroxide but failed to clot milk containing 18 p.p.m. Since, therefore, *Str.* HYW exhibited no unusual sensitivity to peroxide there remained only the possibility that in aerated milk it produced abnormally large amounts of peroxide. Evidence on this point would obviously be more difficult to obtain. Cultures of both normal and sensitive streptococci gave no evidence of peroxide formation in non-aerated steamed milk as judged by the starch and iodide test, which proved capable of detecting 6 p.p.m. of H_2O_2 in the whey from clotted milk. But under conditions (as in aerated milk) in which the *Str.* HYW might form peroxide within or on the surface of its cells, its growth would be inhibited before an amount sufficient to permit recognition by chemical tests could accumulate in the medium. There remained only an indirect test, *i.e.* growth in deep tubes of heated blood agar, in which medium M'Leod and Gordon (6) showed that peroxide formers produced a green colour at the point of limiting oxygen tension. Under these conditions neither *Str.* HYW nor any of the normal lactic streptococci tested gave a positive test for peroxide. There was a tendency for the formation of a ring of slightly darker brown colour about half an inch below the surface of the agar, but since it was not green, and since, moreover, all cultures of streptococci, sensitive or insensitive, gave a similar appearance it could not be considered probable that it had any connection with the sensitivity of the streptococci. There was no difference in the appearance of the cultures when milk was incorporated in the heated blood agar.

Finally, the fact that the sensitive streptococci eventually grew and clotted even aerated milk, although only after a period longer than normal, is not in favour of the peroxide theory, since there is no reason to suppose that any factor would lessen the possibility of peroxide production during a prolonged period of incubation.

POSSIBLE CONNECTION BETWEEN OXIDATION-REDUCTION PHENOMENA AND SENSITIVITY OF STREPTOCOCCI.

The normal sequence of events in a milk culture of lactic streptococci is, first an incubation period in which there is very slow growth of the organisms, very little change in oxidation-reduction potential, and very little acid formation. Then follows a period of rapid growth during which the oxidation-reduction potential falls rapidly and there is vigorous fermentation of lactose. Finally a quiescent state is reached as fermentation slows down and there is little further fall in potential. If, as seems most probable, the fall in oxidation-reduction potential is intimately connected with lactose fermentation and growth of the streptococci, then any factor which interferes with this fall in potential will delay growth of the organisms. It is possible either that too high an initial potential in the milk will delay the initiation of vigorous growth or,

on the other hand, that some factor which interferes with the oxidation-reduction reactions by means of which the organisms obtain energy and incidentally induce a lowered potential, will prohibit their growth for some time. It is not possible to get reliable evidence on slight differences in the oxidation-reduction potential of milk by electrometric means, since the electrode potentials are not sufficiently accurately reproducible. Evidence was, however, sought by the use of oxidation-reduction indicators. Methylene blue remained in the oxidised state in milk which had been heated in steam for half an hour; and consequently no change towards higher potentials could be observed when the milk was aerated. With *o*-chlorophenol indophenol, however, it was observed that reduction of the dye occurred in heated milk, only the surface layers developing a slight colour on standing, whereas when the milk was aerated by being poured once or twice from vessel to vessel the dye reverted to its oxidised state. 2, 6-Dibromophenol indophenol, which occupies a slightly lower position on the E_h scale, maintained its oxidised condition even in steamed milk which had not been aerated. There was thus evidence of a slight lowering of potential when milk was steamed and a subsequent rise of potential on aeration. When, however, a trace of ferrous sulphate was added to heated aerated milk so that a potential low enough to cause decoloration of methylene blue was produced, the sensitive streptococci still refused to grow normally. It did not appear likely therefore that differences in the initial oxidation-reduction potential of the milk were sufficient to account for the behaviour of the sensitive streptococci.

There remained the possibility, mentioned above, that aeration leads to a change in some substance in the medium so that the sensitive streptococci, unlike the normal streptococci, are unable to obtain energy sufficiently rapidly by the normal processes. Evidence on this point was obtained only indirectly. It was found that whey obtained from milk after treatment with rennet was a suitable medium in which to reproduce the original phenomenon. There was in this case of course no possibility of observing the occurrence or non-occurrence of clotting; but titration of the whey with caustic soda after an incubation period gave an indication of the extent of fermentation and growth. The whey was used after adjustment to several *p*H values and also with and without the addition of yeast extract, a well-known stimulant for certain streptococci.

The following account of a typical experiment serves to indicate the general findings. Three portions of whey were adjusted to *p*H values 6·0, 6·5 and 7·0; to half of each portion 10 per cent. of yeast extract was added and to the other half 10 per cent. water. The yeast extract was prepared by digestion of 1 lb. of pressed yeast in 700 c.c. of water followed by filtration. The reaction of the extract was adjusted so that it did not alter the *p*H of the whey to which it was added. Each portion of fluid was distributed in four small conical flasks in quantities of 100 c.c. Four control flasks of the original separated milk were also prepared. All the flasks were steamed for half an hour and

subsequently cooled to 20° C. in water. The fluid in two of each set of four flasks was aerated by pouring back and forth three times into a sterilised beaker. All the flasks were then inoculated with two drops of a 24-hour culture of *Str.* HYW in milk. After incubation at 20° C. overnight the samples were titrated with *N*/10 NaOH and the results expressed as parts per thousand lactic acid.

Table III.

	*p*H	Duplicates not aerated		Duplicates aerated	
Whey	6·0	3·3	3·3	1·7	1·7
	6·5	3·1	2·8	0·7	0·7
	7·0	3·0	2·7	0·5	0·5
Whey + 10 % yeast extract	6·0	4·4	4·5	1·7	1·9
	6·5	4·5	4·6	2·5	2·8
	7·0	4·0	4·0	4·1	4·1
Milk	6·6	7·4	7·3	3·1	2·6

It will be observed that the acidity developed in whey is considerably less than that in milk. Aeration has, however, equally as marked an effect in whey as in milk. The yeast extract has no effect on the properties of the aerated whey at *p*H 6·0. At the higher *p*H values, however, the presence of yeast extract enables the sensitive streptococci to grow in the aerated whey; and at *p*H 7·0 the effect of aeration is entirely eliminated by the extract.

A most interesting observation was made when solutions similar to those used in the above experiment were made with the addition of methylene blue. At the higher *p*H values the whey alone and the whey + yeast extract tended to decolorise the blue while the solutions were hot. On cooling, the blue colour tended to return; and aeration caused the colour to return completely. The solution which decolorised most readily was the whey + yeast extract at *p*H 7·0. Now while there is not necessarily any connection between the development of a reducing potential under the influence of heat on the one hand and under the influence of bacterial enzymes on the other, yet it seems suggestive that the aerated solution which permitted most readily the re-establishment of a reducing potential should be that which most readily permitted growth of the sensitive streptococci. There is, however, the anomaly that aerated whey alone at *p*H 7·0, while it decolorised methylene blue on heating more readily than whey at *p*H 6·0, did not permit any better growth of the streptococci. Furthermore, the addition of yeast extract to milk proved ineffective in eliminating the inhibition produced by aeration.

One more point of information was gained before all the cultures of sensitive streptococci suddenly ceased to exhibit the phenomenon just as the originally isolated cultures had done on a previous occasion. In trials with various synthetic media containing constituents such as peptone and meat extract together with inorganic salts, it was found that the phenomenon of inhibition as a result of aeration was apparently associated specifically with milk, whey, and skim-milk powder. Only those media which contained these latter constituents, in however small a proportion, would exhibit the phenomenon.

Progress towards the elucidation of the mechanism is necessarily slow, since work can only be continued during periods when the cultures are sensitive. Up to the present no method of inducing sensitivity has been discovered; the cultures seem to change spontaneously; and no other property of the organisms has been observed to exhibit a change coincidently with the development or disappearance of sensitivity to aeration. The scanty evidence so far adduced seems to indicate that "sensitivity" means that the organisms have changed in some unknown manner so that they find it difficult to bring about a reducing potential in milk which has been aerated. Accepting the fact of the change in the organisms, the delayed growth may be due, as has been suggested above, to the organisms finding difficulty in dealing with the substances they normally use as hydrogen donators or hydrogen acceptors. In the system

$$\text{Hydrogen donator} \xrightarrow[\text{bacterial enzyme}]{\text{H}} \text{Hydrogen acceptor}$$

it is easy to imagine that a loose combination of oxygen with the hydrogen donator may render the latter less easily available for use by the organisms, thus rendering the development of a reducing potential more difficult. It is tempting to enter into speculation as to the nature of the substance in yeast extract which under some conditions seems to assist the growth of the sensitive bacteria in an aerated medium. Yeast extract is rich in glutathione, and this substance acts most readily as a hydrogen transporter in alkaline solution; but it is too early as yet to suggest that there is any connection between these facts and the action of yeast extract in aerated whey.

No light can be thrown at present on the still more baffling problem presented by the sudden changes between sensitivity and insensitivity exhibited by these peculiar strains of streptococci. It is possible that some factor in the milk, changing with the stage of lactation in the cow or associated with variations in the character of the herbage, which is in turn influenced by weather and season of the year, may induce variations in organisms which are subcultured daily in the milk. Up to the present the phenomenon has manifested itself only in the spring and autumn when the most marked changes in the character of the green feed are taking place; but the connection is not definite enough to afford more than a very slight basis for a working hypothesis, and even then the mechanism is entirely obscure.

From a practical point of view in the manufacture of cheese the phenomenon has an obvious importance. It is known to occur quite frequently in cheese factories, although it is by no means the only cause of starter failure. A case was investigated by Whitehead and Wards(7) in which the effect of aeration of the starter milk seemed to be the main factor, although in that instance the starter culture survived for one generation in the aerated milk and was inhibited only in the second generation. Avoidance of aeration undoubtedly provides a possible remedy for some of the troubles encountered

in practice. In any case, the discovery of the phenomenon brings to light a factor, hitherto unsuspected, which needs elucidation if complete control of starter cultures is ever to be secured.

SUMMARY.

1. Some cases of sudden failure in the growth of cheese starter cultures are described.

2. It is shown that the cause lies in the method of pasteurisation of the milk in which the cultures are grown.

3. Aeration of the milk immediately before the inoculation of the culture is shown with certain cultures to have an inhibitory effect on the growth of the organisms. Normal starter cultures are not affected by aeration of the milk; they may, however, suddenly develop this sensitivity to aeration and may just as suddenly revert to the normal state.

4. The mere absorption of atmospheric oxygen while milk is being cooled after pasteurisation in a wide-mouthed cylindrical vessel may constitute an aeration sufficient to inhibit sensitive cultures of lactic streptococci. Milk pasteurised and cooled in conical flasks plugged with cotton-wool does not reabsorb sufficient oxygen to lead to inhibition, provided that the flasks are not violently agitated.

5. The possible mechanism of the inhibition of the sensitive streptococci is discussed. Evidence is brought forward which tends to show that the action is connected with the oxidation-reduction reactions by means of which the organisms normally obtain their growth energy.

6. The practical significance of the phenomenon in the process of cheese manufacture is emphasised.

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84. THE INFLUENCE OF LACTIC STREPTOCOCCI ON THE RIPENING OF CHEDDAR CHEESE

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(With 4 Figures.)

The problem of the part played by micro-organisms in the ripening of cheese has attracted the attention of workers for many years. It has long been accepted that the lactic streptococci and lactobacilli are the only bacteria likely to affect the normal ripening process of Cheddar cheese, since they are the organisms found most constantly and in the greatest numbers; but their action has still not been clearly defined. There are in general three possibilities.

(*a*) The lactic acid bacteria may influence ripening solely through their production of acid during the making process and in the early stages of ripening. The acid formed by fermentation of lactose most certainly plays a part in determining the precise chemical nature of the cheese curd as a medium for the subsequent development of bacteria during the ripening period. It also influences to a considerable extent the action of the rennet enzyme and the action of any enzymes inherent in the milk.

(*b*) The lactic acid bacteria, by virtue of the proteolytic enzymes which they are known to produce, may play a significant part in the hydrolysis of protein which occurs during the ripening process. This may or may not affect flavour.

(*c*) The part played by the lactic acid bacteria in the protein hydrolysis may be insignificant but they may influence the flavour of the cheese by enzymic reactions on protein decomposition products or on non-protein substances. Products formed from fat and from lactates are possible factors in the development of flavour.

Evidence on all these points has been adduced by various workers; but none of it is conclusive. Evans, Hastings, and Hart (1), Evans (2), and Hucker and Marquardt (3) showed that the addition of specific strains of lactic streptococci to cheese milk influenced the flavour of the cheese. They do not, however, appear to have taken into account the differences in rate of acid production and in final acidity attained during the manufacture of their experimental cheeses; and it seems possible that flavour may have been influenced more by the effect of these differences on the action of the rennet enzyme and on the development of the cheese bacteria in general than by the specific properties of the added bacteria. It has never yet been possible to eliminate from the

cheese all bacteria except those added as starter culture, so that unless two cheeses are made under exactly similar conditions of acid development in the vat, it is not possible to ascribe differences between them simply to the different properties of the added bacteria and to ignore the possible effect of differences in acidity on the action of enzymes and on the subsequent development of organisms such as lactobacilli. Barthel(4) and Barthel and Sandberg(5) demonstrated that lactic streptococci could decompose casein slowly at the temperatures usually employed for the ripening of cheese. Later, Barthel and Haglund(6) performed experiments on the ripening of Gouda type cheese. They took into account the possible effect of acid itself and came to the conclusion that although differences in acidity induced differences in the rate of ripening, differences in the numbers of lactic streptococci in the milk at the time of adding the rennet had a much greater influence in inducing differences in the rate of ripening. Barthel and Haglund(7) then tried to influence the rate of ripening by the use of starters consisting of pure strains of streptococci with known proteolytic power, taking into account at the same time the acidity and bacterial content of the milk at the time of renneting. They obtained negative results, and attributed this failure to the fact that the pure strains of streptococci used had a lower rate of development and lower acidifying power than the mixed cultures used in their previous experiments. The results of these experiments on Gouda type cheese are difficult to interpret in a consideration of Cheddar cheese owing to differences in the making processes. Barthel and his co-workers apparently took into account only the acidity *at the time of renneting* and did not determine at what rate acidity developed in the curd from that time onwards. It would seem more reasonable to take account of the acidity of the curd at the end of the cheese-making process when the most rapid changes in acidity are over. Thus in Barthel's experiments, two vats of milk might have had equal numbers of organisms and equal acidity at the time of renneting, but acid may have developed thereafter at quite different rates especially where pure cultures of streptococci were being compared with mixed cultures. Kelly(8, 9), working on the rate of ripening of Cheddar cheese, came to the conclusion that acid production was the main function of a starter culture and that different types of streptococci did not induce differences in the rate of ripening apart from the acidity factor. In his experiments, however, he used raw milk; and there is considerable doubt according to his own data whether the pure cultures added ever did predominate in the cheese curd.

The original object of the present work was to determine whether differences in rate of ripening could be observed when pure strains of streptococci of differing proteolytic powers were used as starters in the manufacture of Cheddar cheese. An attempt was made to arrange the experiments so that various vats of similar milk, inoculated with the different streptococci and mixtures of streptococci, developed acid at the same rate throughout the process of cheese manufacture. Unfortunately it proved impossible to realise

this in those cases where organisms with a low proteolytic power were being compared with organisms of marked proteolytic power. It was found impossible to isolate streptococci with low proteolytic power which would produce acid at a sufficiently rapid rate. This is in accord with the findings of Anderegg and Hammer (10). It was therefore necessary in some cases to accept the differences in rate of acid production and to make allowance for this factor in a consideration of the results. The cheeses made were analysed chemically according to the technique (somewhat modified) developed by Eagles and Sadler (11) from the previously described protein partition methods of Wasteneys and Borsook (12). The work reported here is open to criticism from the point of view of the small number of cheeses on which the conclusions are based; but the results are so consistent from some angles that we consider they are of value as a basis for further development of the work.

Methods used for examination of milk cultures of streptococci.

(*a*) *Determination of proteolytic power of streptococci.*

30 g. of chalk were added to 1000 c.c. of separated milk and the mixture was autoclaved at 12 lb. for 30 min. An amount of a 24-hour culture of streptococci in milk, equivalent to the amount used as starter in the cheese vat, was added and the flask containing the culture was incubated at 20° C. for 1 month. After the absence of contaminating bacteria had been demonstrated, the flask was made up to its original weight by addition of distilled water. The chalk precipitate was allowed to settle and then 500 c.c. of the milk, decanted from the flask, were treated with 100 c.c. of trichloracetic acid. After being allowed to stand for 1 hour, the protein precipitate was filtered off and two 200 c.c. portions of the filtrate were evaporated down on a boiling water bath, the object being both to remove the excess of trichloracetic acid and to concentrate the solution. The two portions were finally made up to 100 c.c. each, and duplicate 10 c.c. amounts from each were taken for determination of nitrogen by the Kjeldahl method. The solutions contained all the non-protein N.

To the remainder of each solution were added 30 g. of anhydrous sodium sulphate. The mixtures were maintained at 35° C. with occasional shaking during 1 hour which resulted in a precipitation of the proteose. The liquids were filtered through filters maintained at 33° C. 50 c.c. of each filtrate were made up to 100 c.c. at room temperature and duplicate 25 c.c. amounts from each were taken for Kjeldahl determination. The results gave a measure of the amount of peptone N + subpeptone N still in the fluid; and the amount of proteose N precipitated could be calculated by difference. The necessary corrections had to be made for volume changes at the different temperatures used.

25 c.c. of each peptone + subpeptone solution were treated with 25 c.c. of 2·21*N* NaOH and 125 c.c. of tannic acid solution (200 g. anhydrous Na_2SO_4

dissolved in 0·1*N* H_2SO_4; made up to 1000 c.c. with 0·1*N* H_2SO_4 and 200 g. tannic acid added). The mixtures were allowed to stand at 20° C. during 3 hours in order to permit complete precipitation of the peptone. Filtration then gave clear liquids of which duplicate 50 c.c. portions were subjected to a Kjeldahl determination. From the results the subpeptone N could be calculated.

(*b*) *Determination of volatile acid produced in milk cultures.*

The streptococci were allowed to grow in sterilised separated milk for 3 or 4 days. Then 150 g. of the culture were weighed out; 7·5 c.c. of 2*N* H_2SO_4, 5 g. Na_2SO_4 and a little paraffin wax were added and the mixture was subjected to steam distillation. The rate of distillation was standardised so that 1000 c.c. of distillate were collected in 80 min. The results were expressed as c.c. of *N*/10 NaOH required to neutralise the first litre of distillate to phenolphthalein.

Methods used in the manufacture and examination of cheese.

Preparation of starter.

Throughout the work four cultures of single strains of lactic streptococci were used singly or combined as starters. The quantities of starter required for addition to the vats were prepared in the laboratory by growth of the organisms in steamed separated milk. Two strains (A and D) grew rapidly enough to clot milk in 24 hours at 20° C., but the other two strains (B and C) needed incubation at 30° C.

Manufacture of the cheese.

The milk was obtained from the Massey Agricultural College herd. It was regularly of excellent quality, having a low bacterial count and a methylene-blue reduction time of over 7 hours. It was pasteurised at 150° F. by the flash method before being run into the cheese vats. The technique of cheese manufacture was normal. The main particulars of the process will be given later in dealing with the results. The cheeses (usually of 40 lb. weight) were allowed to ripen in a curing room maintained constantly at 60° F.; they were turned daily.

Method of sampling.

At each examination two large plugs extending to the centre were taken from each cheese. One plug was drawn half-way up the side of the cheese, and the other one-third of the way up. Half an inch of the rind end of each plug was rejected. A small portion of each plug was reserved for bacteriological examination.

Determination of the nitrogen partition.

The two plugs were ground to a paste in a mortar and the total N content was determined on two 1 g. samples. 51 g. of the paste were gradually triturated with 400 c.c. of distilled water at 50–55° C. The resulting emulsion contained

in a 500 c.c. graduated flask was maintained at 37° C. with occasional shaking during 2 hours. The liquid was then made up to 510 c.c. and the fat layer on the surface was siphoned off. The protein insoluble in water was filtered off and the soluble N was determined on duplicate 10 c.c. portions of the filtrate. 350 c.c. of the filtrate were treated with 70 c.c. of 20 per cent. trichloracetic acid, and the remainder of the procedure was the same as that described for the milk cultures. A *p*H determination was carried out electrometrically on the aqueous solution of the cheese prepared as described above.

Bacteriological examination of cheese.

The usual technique was followed in plating out the cheese for estimation of the total number of bacteria present. A gelatine medium containing whey, yeast extract, peptone, and phosphate was used. Smears made from thirty colonies picked from each plate were examined under the microscope in order to estimate roughly the relative numbers of streptococci and lactobacilli.

Results.

Characteristics of the lactic streptococci used as starters.

All four strains of streptococci were members of the *Str. cremoris* group according to the classification of Orla-Jensen (13). They did not ferment maltose, dextrin, or salicin; nor did they form any significant amount of volatile acid during their growth in milk even after an addition of 0·4 per cent. of citric acid to the medium. The only significant differences between them were in the rate and extent of acid formation, and in the extent and type of proteolysis produced in milk. Table I gives the results of analyses of chalk-milk cultures

Table I. *Proteolysis produced by starter organisms in chalk-milk.*

Organisms	H_2T	A	B	C	A+B	A+C	D	D+B	D+C
	Nitrogen formed as percentage of total nitrogen.								
Non-protein N	16·2	23·9	20·4	1·9	21·3	23·1	2·0	8·9	2·2
Proteose N	−0·6	1·7	4·3	−0·3	1·7	2·2	0·2	0·3	0·1
Peptone N	9·0	10·9	8·4	0·2	9·7	10·0	0·8	5·6	1·0
Subpeptone N	7·8	11·3	7·7	2·0	9·9	10·9	1·0	3·0	1·1
	Nitrogen formed as percentage of non-protein nitrogen.								
Proteose N	−4·0	7·3	21·4	−15·7	8·5	9·5	10·0	3·3	4·6
Peptone N	55·6	45·6	41·0	10·4	45·2	43·2	40·0	62·9	45·4
Subpeptone N	48·4	47·1	37·6	105·3	46·3	47·3	50·0	33·8	50·0

of the single strains and of the mixtures of strains which were subsequently used in the cheese vat. The results obtained from a normal commercial starter culture (H_2T) are included for purposes of comparison. The only significant anomaly to be noted in the table is that the extent of proteolysis produced by organisms D and B together is less than that produced by organism B alone. It may be necessary in the future to investigate more closely the possible interactions between lactic streptococci in mixed culture so far as proteolysis is concerned.

Experiments in cheese manufacture.

Two main experiments were carried out. The idea in the first was to use proteolytic organism A as the main starter and to try the effect of extra additions to the cheese milk of (*a*) a second proteolytic organism, and (*b*) a non-proteolytic organism. In the second experiment a non-proteolytic organism (D) was used as the main starter and the same extra additions were made. On the day on which the second experiment was performed an extra vat of milk was treated with organism A alone as a starter in order that it might form a link between the two experiments. Table II gives the main particulars of the cheese-manufacturing process throughout the two experiments. It will be observed that in the first experiment acidity developed in all three vats at approximately the same rate, in spite of the fact that an extra 2 per cent. of starter culture was present in vats 2 and 3. The impossibility of finding a non-proteolytic organism which would produce acid at a sufficiently rapid rate has already been mentioned; this is evidenced in the details given for the second experiment. Organism D was chosen as being one of the most active of the non-proteolytic organisms. In spite of the use of 5 per cent. of culture in vat 5, the cheese-making process was prolonged and acidity was very low at the time of salting. The presence of an extra 2 per cent. of proteolytic organism B in vat 6 increased the rate of acid production markedly, while the extra 2 per cent. of non-proteolytic organism C in vat 7 had practically no effect. In these experiments and in other similar ones the general finding has been that acid-producing power is linked with proteolytic power.

Analyses of experimental cheeses.

In order to save space the detailed results of the chemical examination of the cheeses are not given here; the results are depicted in graphs which show all the essential points.

Fig. 1 shows the progress of protein breakdown for the three cheeses of the first experiment; the graphs are plotted from the results of examinations carried out at weekly intervals. It will be observed that there was no significant difference between the three cheeses at any stage of the ripening process in spite of the fact that many more lactic streptococci had been present in the milk in vats 2 and 3 at the time the rennet was added. Direct microscopic counts on the milk in the cheese vats after the addition of the starter cultures proved that vats 2 and 3 contained at least twice as many organisms as vat 1, and bacteriological examination of the cheeses on the day after manufacture showed an even greater disparity. The results are therefore in disagreement with those of Barthel and Haglund who, as previously mentioned, obtained an increased rate of ripening correlated with an increased number of lactic streptococci in the milk at the time of renneting. In our experiment the equal rates of ripening of the cheeses corresponded with the similarity in action of the starters in chalk-milk cultures and with the equal rates of acid development

Table II. *Manufacturing details.*

Cheese No.	Starter	Percentage casein in milk	Percentage fat in milk	Rennet	Acidities as percentage lactic acid: When vat filled	When renneted	*At running of whey	*When curd dry (piled)	At salting	Times in hours and minutes: From filling vat to renneting min.	From renneting to running hr. min.	From renneting to drying (piling) hr. min.	From drying to salting hr. min.	Percentage salt to curd
1	1·5 % A	2·61	4·1	4 oz. per 1000 lb. milk	0·17	0·18	0·17	0·27	1·01	42	2 55	3 4	3 0	2
2	1·5 % A + 2·0 % B	2·61	4·1	,,	0·17	0·19	0·18	0·275	0·94	46	2 48	2 54	3 17	2
3	1·5 % A + 2·0 % C	2·61	4·1	,,	0·17	0·185	0·18	0·28	0·94	46	2 52	3 1	3 10	2
4	2 % A	2·73	4·5	,,	0·16	0·17	0·185	0·33	—	31	3 21	3 32	2 11	2¼
5	5 % D	2·73	4·5	,,	0·16	0·18	0·16	0·18	0·49	33	4 14	4 20	3 31	2¼
6	5 % D + 2 % B	2·73	4·5	,,	0·16	0·20	0·185	0·26	0·82	33	3 5	3 14	3 41	2¼
7	5 % D + 2 % C	2·73	4·5	,,	0·16	0·195	0·17	0·195	0·51	32	4 13	4 19	3 31	2¼*

* Acidity of free whey, not acidity of whey inside curd particles.

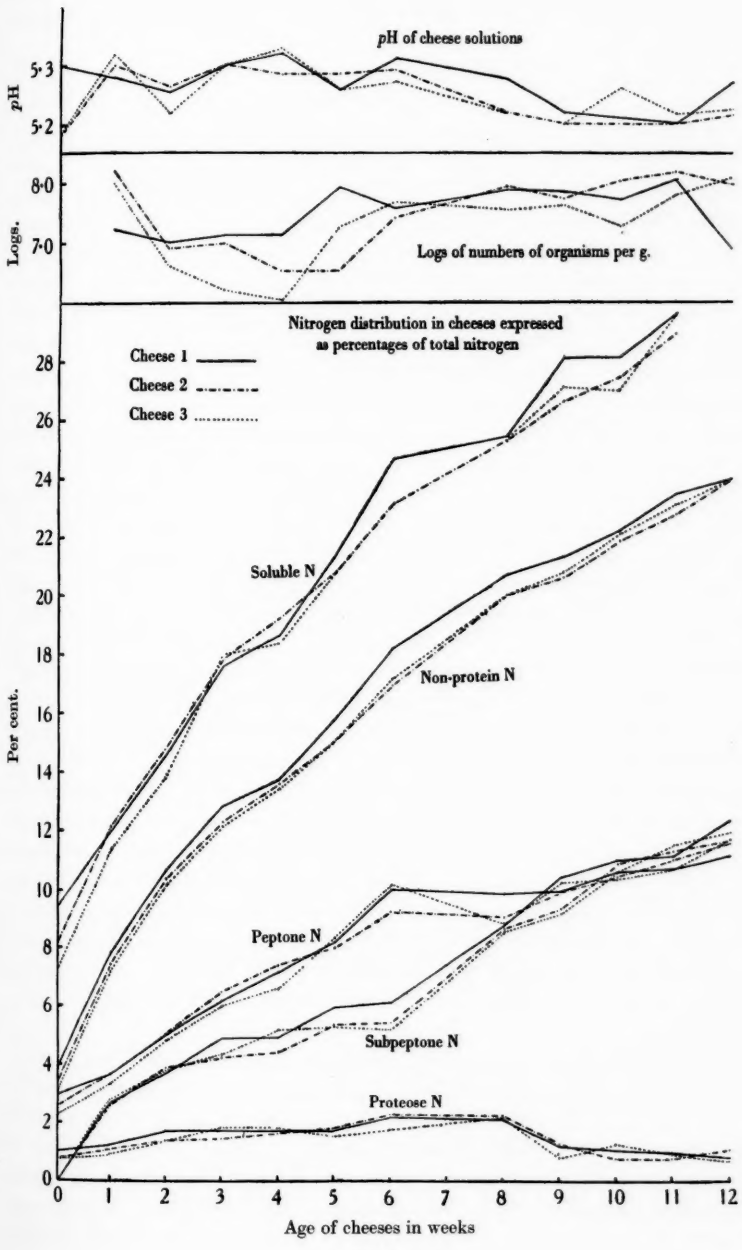
pH of cheese solutions
pH
5·3
5·2
8·0
7·0
Logs.
Logs of numbers of organisms per g.
Nitrogen distribution in cheeses expressed as percentages of total nitrogen
Cheese 1
Cheese 2
Cheese 3
Soluble N
Non-protein N
Peptone N
Subpeptone N
Proteose N
Per cent.
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
0 1 2 3 4 5 6 7 8 9 10 11 12
Age of cheeses in weeks

Fig. 1.

in the three vats. The fact that organism B was proteolytic whereas organism C was non-proteolytic makes the lack of difference in the rates of ripening all the more noteworthy.

With regard to the general character of the graphs, it will be observed that the soluble N and non-protein N curves indicate smooth continuous protein hydrolysis. Later in the maturing process the subpeptone curves rise more steeply than the peptone curves, and the two types of curves intersect at about 9 weeks. Proteose N shows a rise to a maximum and then a slow decrease. The general trend of all the curves confirms the results of several other examinations of normal Cheddar cheeses made with commercial starter cultures.

Fig. 2 illustrates the results obtained in an examination of the four cheeses made in the second experiment. The subpeptone curves are not included in this case in order to avoid confusion due to overlapping. The values for subpeptone N may be obtained by subtraction of proteose N + peptone N from non-protein N. Cheese 4, made with organism A as starter, forms a link between the two experiments. Although cheeses 1 and 4 were made on different days and with slightly different percentages of starter, they exhibit a close similarity in their nitrogen partition. The main point which emerges from a consideration of the graphs for the second experiment is that the curves for the two cheeses, which progressed normally during the making process, are differentiated sharply from the curves for the two "slow" cheeses. This also applies to the curves showing *p*H values which are included in these graphs. The large initial difference of approximately 0·4 *p*H unit sinks to about 0·15 unit as ripening progresses, but a definite difference is maintained throughout the period of observation. The form of the curves for protein breakdown is the same both for the normal and the "slow" cheeses, but the difference in total extent of breakdown is marked throughout. Although the mixture of organisms D and B showed less proteolysis in chalk-milk than organism A, the curves for the corresponding cheeses show a close similarity, at any rate so far as soluble N, non-protein N, and peptone N are concerned, which correlates with the rates at which acid was produced in the vats. Throughout the results of this experiment there is a strong suggestion that the nitrogen breakdown, so far as the broader fractions are concerned, is governed much more by the rate of acid production and final acidity reached in the cheese vat than by the numbers or proteolytic properties of the lactic streptococci added to the milk.

On the other hand, inspection of the curves renders it apparent that the bacteria in the starter may possibly be responsible for a difference in partition between proteose N and subpeptone N. The general form of the proteose curve for cheese 4 is quite different from that for cheese 6. The proteose N decreases more rapidly in cheese 4 as the cheese ages; and consequently there is a correspondingly increased rise in subpeptone N. Since these differences between cheeses 4 and 6 occur long after the disappearance of lactic streptococci from the cheeses we have to consider two possible causes.

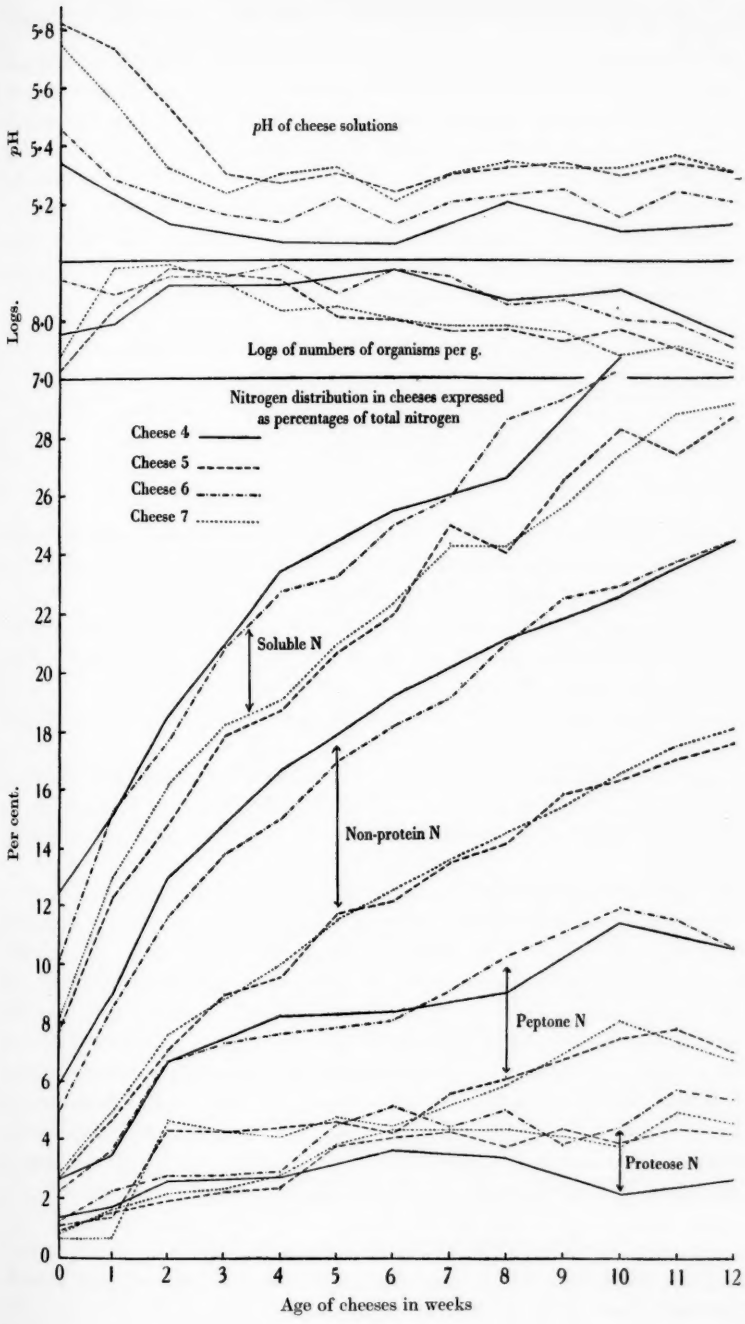
pH
5·8
5·6
5·4
5·2
pH of cheese solutions
Logs.
8·0
7·0
Logs of numbers of organisms per g.
Nitrogen distribution in cheeses expressed as percentages of total nitrogen
Cheese 4
Cheese 5
Cheese 6
Cheese 7
Per cent.
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Soluble N
Non-protein N
Peptone N
Proteose N
0 1 2 3 4 5 6 7 8 9 10 11 12
Age of cheeses in weeks

Fig. 2.

(*a*) The effect may be due to the continued action of enzymes liberated on the death of the streptococci. Virtanen and Lundmark (14), and Virtanen (15) have shown that the enzymes of *B. casei* ϵ continue to bring about proteolysis after inhibition or death of the organism. Some experiments carried out during the present work served to show a similar action by a typical commercial starter culture. Thus in Table III the first column gives the nitrogen partition in a chalk-milk culture after incubation at 20° C. for 5 weeks, at which time all the streptococci proved to have died out. The second column gives the nitrogen partition of a portion of the same culture 1 month later.

Table III. *Proteolysis produced by starter* H_2T. *Nitrogen formed as percentage of total* N.

	1	2 (1 month later)
Non-protein N	16·2	21·2
Proteose N	−0·6	−1·5
Peptone N	9·0	11·1
Subpeptone N	7·8	11·8

It will be observed that the type of breakdown remains almost the same, but that the extent of proteolysis is increased, proving the continued action of the enzymes after death of the organisms.

(*b*) The effect may be due actually to proteolytic action by lactobacilli whose development is affected by the products left by the streptococci or formed by the rennet enzyme.

There is no definite evidence at present to suggest which of these two possibilities is correct.

Fig. 3 gives graphs of the various nitrogenous constituents of the four cheeses of the second experiment plotted as percentages of the soluble N. The differences between the normal and "slow" cheeses are still more strikingly shown.

The nitrogen partition is, however, seen in most detail in Fig. 4, where the types of nitrogen are plotted as percentages of the non-protein N. The most interesting changes are those shown by the curves for peptone N. Initially widely separated, the curves suddenly exhibit an enormous rise and the points for all the four cheeses almost coincide. It will be shown later that this coincidence happens at the time at which the replacement of the streptococcal flora by the lactobacilli occurs in the cheeses. Thereafter, the curves for normal and "slow" cheeses separate and pass through a minimum. The curves for proteose N show the usual maximum which occurs at the minimum for the peptone N. The curves for subpeptone N (not shown on the graphs) show a sharp fall to a minimum corresponding to the maximum in peptone N.

Bacteriological examination of the cheeses.

Curves plotted from the total bacterial counts of the cheeses of both experiments are shown on the corresponding nitrogen partition graphs. In view of the well-known large variations in the numbers of bacteria from point

to point in Cheddar cheese, it is doubtful whether any really reliable conclusions can be drawn from these curves. There appears, however, to be an anomaly between the results of the two main experiments. In the first

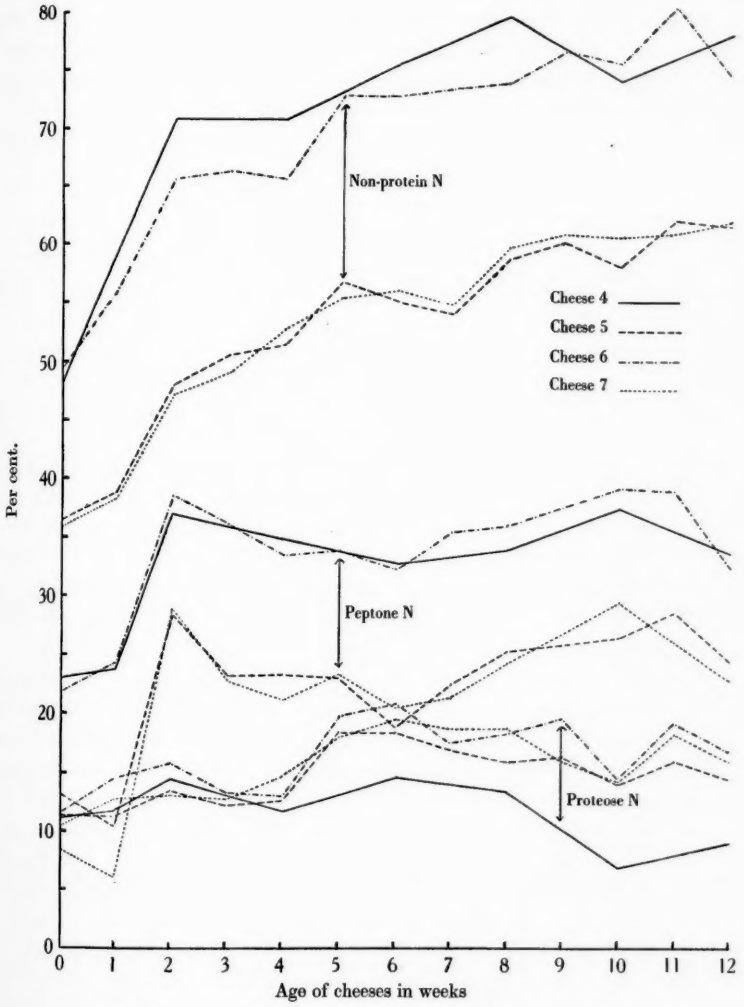

Fig. 3. Nitrogen distribution in cheeses expressed as percentages of the soluble nitrogen.

experiment the numbers of bacteria tend to show a decrease during the first 3 weeks followed by a rise to a relatively constant level. In the second experiment the numbers show an early rise followed by a gradual fall. The "slow" cheeses 5 and 7 show lower counts than the normal cheeses on the day after

manufacture. Possibly this is explained by the absence from those vats of any proteolytic streptococci which might assist the growth of the non-proteolytic organisms. The "slow" cheeses also show consistently lower counts after the first 3 or 4 weeks.

Estimation of the relative numbers of streptococci and lactobacilli was made only during the second experiment. Table IV gives the results as percentages of lactobacilli to total organisms, estimated from examination of thirty colonies in each case.

Table IV.

Cheese	2 days	7 days	14 days
4	0	10	100
5	0	8	100
6	0	10	100
7	20	90	100

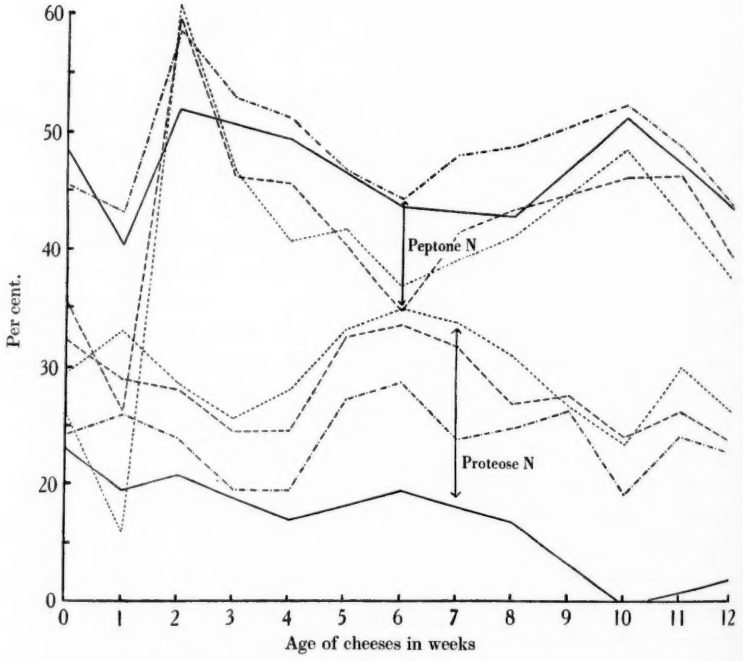

Fig. 4. Nitrogen distribution in cheeses expressed as percentages of non-protein nitrogen.

Reference to Fig. 3 shows that the main difference between the "slow" cheeses 5 and 7 in the first few days of ripening is that the latter has a lower proportion of the soluble N in the form of peptone N. This is almost entirely accounted for by a correspondingly greater proportion in the form of sub-peptone N. It is possible that this difference between the two cheeses may account for the earlier appearance of the lactobacilli in cheese 7. This would

be in accord with the work of Davis and Mattick(16), who found that streptococci grow best in solutions containing the more complex nitrogen compounds while lactobacilli prefer simpler nitrogen compounds. In unfinished experiments by the present authors it has been found that cheeses containing double the usual amount of rennet show larger proportions of proteose and peptone N and a delayed replacement of streptococci by lactobacilli. It is possible therefore that the type of breakdown in the lower protein products, initiated by specific starter organisms, may have a significant influence on changes in the flora of a cheese as it ripens.

Commercial quality of the experimental cheeses.

All the cheeses were examined at intervals by two experienced judges of Cheddar cheese. The three cheeses of the first experiment exhibited no marked differences throughout the ripening period, and, at an age of 6 months, they were pronounced typical ripe cheese of good quality. The four cheeses of the second experiment showed differences in the early stages of ripening. Cheeses 5 and 7 were somewhat "sweet," as was to be expected from the low acidities which were developed in the vats. The differences eventually disappeared, however, and remarkably enough, at an age of 3 months all four cheeses were practically equally good in quality. Again at an age of 6 months there were no significant differences between the cheeses, although cheeses 4 and 6 were preferred slightly to cheeses 5 and 7 which had been "slow" during the making process. It is indeed surprising that there should have been so little difference in commercial quality between the four cheeses of the second experiment, in view of the different rates of acid production in the vat and the wide differences in extent of protein breakdown shown in the chemical analyses. It would be unwise to generalise from so few cheeses, but the question does arise as to whether commercial quality, especially in so far as characteristics like "body" and "feel" of cheese are concerned, may not depend on some insoluble protein constituents rather than on the water-soluble portion of the cheese. The water-insoluble portion comprises about 70 per cent. of the protein material of the cheese; and it seems possible that the soluble products may vary within relatively wide limits without markedly affecting the general character of the cheese. There is no evidence in these experiments to suggest that the protein degradation products affect the flavour to a significant extent. Examinations of commercial cheeses have further shown that, quite often, great differences in flavour are exhibited between cheeses with very similar nitrogen partition characteristics. It is possible that in the experiments detailed above, the high quality of the milk used may have accounted for the good flavour of the "slow" cheeses. Under commercial conditions the presence of undesirable organisms in the milk might have led to greater differences in flavour, since such organisms would not have been held in check in the early stages of ripening while the cheeses were still "sweet."

On the whole it seems probable that a normal rate of acid production in

the vat leads in general to a cheese which ripens normally throughout; and this factor seems to be much more significant than the relative proteolytic powers of different strains of streptococci used as starters. It remains to be determined whether the streptococci influence flavour by processes not directly connected with proteolysis.

SUMMARY.

1. The process of ripening in experimental cheeses was followed by periodic estimations of the amounts of various types of protein degradation product formed.

2. No connection between the type of nitrogen partition and the commercial quality of the ripened cheese could be traced.

3. The proteolytic powers of the various pure cultures of streptococci used as starters in the cheese vats seemed to have little influence on the rate of proteolysis in the cheeses during the ripening period. The main trend of the protein breakdown was governed by the rate and total extent of acid formation in the milk and curd during the manufacturing process. This suggests that the rennet enzyme is the principal proteolytic agent, and that the most significant function of the streptococci is the formation of acid which in turn influences the rate of action of the rennet enzyme.

4. No specific connection between the numbers of streptococci present in the cheese milk at the time of renneting and the rate of ripening of the cheese could be traced.

5. Some evidence was found tending to suggest that minor differences in the type of protein degradation occurring during the ripening period influenced the changes of bacterial flora in the cheese. Lactobacilli appeared to develop earlier where the proportion of simpler protein derivatives was high.

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85. NON-PATHOGENIC HAEMOLYTIC STREPTOCOCCI OCCURRING IN MILK

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(With 1 Plate.)

Streptococci, the deep colonies of which in blood agar lie in clear haemolytic zones, are commonly present in the milk of individual cows and hence in market milk. These beta-haemolytic streptococci can be arranged in three classes:

(i) Those which produce chronic inflammatory changes in the mammary tissue but which are without danger to human health. These belong to the group known as *Streptococcus agalactiae*, recognised as the common cause of contagious mastitis of cows.

(ii) Streptococci which may be termed *Str. pyogenes*, in view of their general resemblance to similar organisms found in man. These organisms also cause mastitis, but there is recent evidence (P. R. Edwards(1)) that they are not all of the same kind. Some are derived from man, and the milk when consumed in the raw state may then act as a source of disease (septic sore throat or scarlet fever) in the human population. Others can be distinguished as true animal streptococci and do not produce disease in man.

(iii) Streptococci, such as are described in this article and which according to the evidence are non-pathogenic for cow or man.

Since the discovery that there may occur in milk streptococci which are pathogenic for man, differentiation has become a matter of great importance. In this work investigators have been mainly concerned with the first two classes mentioned, and these, as a rule, can now be distinguished without much difficulty. There is still the chance that some confusion may arise between the second and third classes, and the main purpose of this paper is to place on record the characters of certain streptococci which fall into the third class, in order that their existence in milk may be more widely recognised.

F. S. Jones(2), in a paper dealing with the origin and significance of streptococci in market milk, drew attention to a group of streptococci which produced relatively small colonies in blood agar surrounded by wide zones of haemolysis. Surface colonies were also small, rarely exceeding 1 mm. in size, and rested on a wide bed of haemolysis. Growth in broth took place very slowly at first, though it was rather better when serum was added. The organism fermented glucose, lactose and saccharose but not salicin, mannite, inulin or raffinose. The amount of acid formed from fermentable sugars, however, was distinctly less than in the case of beta-haemolytic mastitis streptococci, the final *p*H in a serum broth medium containing 1 per cent. glucose being 5·0–5·3. Saccharose was not always fermented at the first attempt, and one strain consistently failed to ferment this sugar. Litmus milk

was acidified and coagulated, though not firmly. Jones refers to these organisms as "low-acid-producing streptococci," and they were recovered from about half of twenty-six samples of mixed milk from a certain herd. When the milk of individual cows was examined only a few samples were found to contain these organisms and the numbers present were comparatively small. There was no indication that they were responsible for disease of the udder and the strains were non-pathogenic for mice and rabbits. From a few cows in the herd in question Jones also isolated strongly haemolytic streptococci, which were similar to the low-acid-producing strains in all respects except that they fermented salicin and mannite.

Brown, Frost and Shaw(3) studied the types of beta-haemolytic streptococci present in samples of milk from five certified herds comprising about 900 cows and this work was continued by Frost, Gumm and Thomas(4). The last-named authors report that during a period of 18 months 1304 samples of mixed milk from groups of ten cows were examined and beta-haemolytic streptococci were found in 903 (69 per cent.) of the samples. Out of 1065 beta colonies, however, only 394 were lytic in a tube test carried out by mixing the supernatant fluid from 24-hour cultures in veal broth containing a little serum with an equal volume of 5 per cent. rabbit blood corpuscles, the readings being taken after incubation at 37° C. for 2 hours. They record the results of the examination of 383 cultures, which were lytic under these conditions. All of them except four could be classified as shown in Table I, the designations used being those of Holman, except that a new species, *Str. asalignus*, similar in some respects to *Str. anginosus*, has been added. The unsuitability of the name, *Str. infrequens*, for the organism found in milk is recognised.

Table I.

Action on								
Lactose	Saccharose	Salicin	Mannite	Sodium hippurate	Final *p*H 1 % glucose broth	Capsule	Designation	No. of strains
+	+	+	−	+	4·4–4·9	−	*Str. mastiditis*	186
+	+	+	+	Slight +	4·5–5·0	−	*Str. infrequens*	114*
+	+	−	−	+	4·6–5·1	−	*Str. asalignus*	64†
−	−	−	−	+	4·7–5·2	−	*Str. subacidus*	8
+	+	+	−	−	4·9–5·2	+	*Str. epidemicus*	4
+	+	−	+	Slight +	5·1–5·9	−	*Str. haemolyticus*	3

* These cultures differed from the classical description of this organism in producing rather more acid from glucose and in the slight tendency to split sodium hippurate.

† Fifteen of these strains differed from the remaining forty-nine fermenting saccharose slowly or not at all and in tending to give a slightly higher *p*H in glucose broth.

Our interest in the streptococci, described as belonging to the third class, was first aroused in 1932 at the time of a mild epidemic of sore throat in a boys' school in Surrey, which the Medical Officer thought might be due to contaminated milk[1]. It was reported that haemolytic streptococci in small numbers had been found in milk supplied to this school, and an examination of the milk of the individual cows in two suspected herds was accordingly undertaken[1]. Haemolytic streptococci were present in the milk of a number of the animals, but they were of the kind ordinarily met with in mastitis. One cow, however, was isolated temporarily, because her milk although quite normal in appearance contained streptococci which were actively haemolytic

[1] We are indebted to Mr F. W. Medlock, M.R.C.V.S., for putting us in touch with these herds and to Dr R. J. V. Pulvertaft for a culture obtained from the milk.

when the supernatant fluid from serum broth cultures was mixed with equal parts of a 5 per cent. suspension of rabbit cells. This organism, however, fermented salicin and mannite and was also different in other respects from *Str. pyogenes*. The culture originally isolated from the mixed milk, and which had given rise to some suspicion, was found to be similar to the one isolated from this cow and corresponded to the organism designated *Str. infrequens* in the work of Frost, Gumm and Thomas (4). In consequence of this incident, a look-out has been kept for streptococci with similar haemolytic characters in the milk samples received in large numbers at this Institute. Colonies were picked to tubes of 1 per cent. serum broth, and after 24 hours the supernatant fluid was tested for lytic properties by mixing it in equal quantity with a 5 per cent. rabbit cell suspension in accordance with the technique recommended by Brown, Frost and Shaw (3). Cultures which were actively haemolytic under these conditions were retained for further study. In this way fifty-eight strains have been collected, and these fall into two groups:

(1) Thirty-five strains from four herds, corresponding to the low-acid-producing streptococci of Jones. We abstain from giving a name to this organism and are content to refer to it in this paper as the "low-acid *Streptococcus*."

(2) Twenty-three salicin- and mannite-fermenting strains, from thirteen herds, which will be referred to as *Str. infrequens*, since they probably correspond to the milk streptococci thus described by Frost, Gumm and Thomas. They also appear to correspond to the mannite-fermenting streptococci mentioned by Jones.

Some of the differential characters of these and other haemolytic udder streptococci are summarised in Table II.

Table II.

	Haemolysis									Pathogenicity	
	Deep colony in blood agar			Milk containing		Fermentation					
Group	Colony/zone ratio	Diameter of zone mm.	M.H.D.	Litmus	Methylene blue	Saccharose	Salicin	Mannite	Sodium hippurate	Mouse and rabbit	Udder
Streptococcus of chronic mastitis	1/2 to 1/6	1–3	>1·0	Ac. clot reduction	—	+	+ or −	−	+	−	+
Str. pyogenes	1/5 to 1/10	2–4	0·01	Ac. liq.*	Slight reduction	+	+	−	−	+	+
Low-acid *Streptococcus*	1/12 to 1/15	3–4	0·01	Ac. liq.	—	−	−	−	−	−	−
Str. infrequens	1/12 to 1/15	3–4	0·01	Ac. liq.	—	− or +	+	+	−	−	−

* Some strains produce a soft clot within 5 days.

OCCURRENCE IN MILK.

Samples of mixed milk from twenty-five farms were examined by plating dilutions of the cream, whole milk or deposit, in ox-blood agar containing crystal violet to a concentration of 1/200,000. Milks from a number of these farms were examined six to nine times. Streptococci with the characters of

low-acid streptococci or *Str. infrequens* were found in the milk from nineteen of these farms, though not on every occasion or in large numbers. Usually, less than twenty colonies developed in the plate seeded with 0·1 c.c. of cream or with the deposit from 10·0 c.c. milk, though sometimes as many as forty to fifty could be counted. The nature of the streptococci was ascertained in the case of the milk samples from thirteen of the nineteen farms, colonies with the characteristic wide zones being picked after 48 hours' incubation. In this series *Str. infrequens* was the more widely distributed organism, being present in the milk from thirteen herds, whereas the low-acid *Streptococcus* was isolated from the milk of four herds only.

Milk samples from the 150 individual cows making up one of these four herds were then examined, with the result that low-acid streptococci were cultivated from twenty-nine quarters of twenty-one cows. A re-examination showed, however, that they could not be isolated with constancy from these quarters. The number of colonies developing in plates seeded with the centrifuged deposit from 10 c.c. fore milk varied from 2 to 8 (mean 5) in fourteen quarters and from 10 to 100 (mean 50) in fifteen quarters. The milk was normal in appearance in all cases, and there was no evidence that the organism caused any disturbance in the gland.

Haemolytic streptococci of these two kinds do not seem to be present in the milk of every herd, since we failed to find them in samples from about 300 cows belonging to eight other herds.

The low-acid-producing milk Streptococcus.

Cultural properties. In deep ox-blood agar plates seeded with centrifuged milk deposits containing this organism, growth occurs in the form of pin-point colonies, surrounded by broad, clear zones. Colonies are often not apparent until the plates have been incubated for 36–48 hours, whereas the streptococci of chronic mastitis and *Str. pyogenes* usually produce recognisable colonies within 24 hours.

Morphological characters. In broth with or without 1 per cent. horse serum or 1 per cent. glucose, growth appears within 24 hours in the form of a faint turbidity and after 2 or 3 days there is slight sedimentation. On microscopic examination, the organisms are seen to be relatively minute and smaller than *Str. pyogenes* or the streptococci of chronic mastitis. They occur singly, in pairs or in very short chains, and are Gram-positive. In 1 per cent. serum broth or in blood agar cultures incubated overnight in a moist atmosphere, capsules could not be demonstrated by the india ink method.

Colony morphology. On agar containing blood or serum a thin growth appears on the most thickly seeded areas within 24 hours, but isolated colonies are not fully developed until the second day; they are then tiny, low convex and have entire edges and a glossy surface. If the cultures are left at room temperature for a few days, the only change is the appearance of a slight granularity. Apart, however, from their size, colonies are not very different

in appearance from those of *Str. pyogenes*. The characters mentioned serve to differentiate them from the irregular or confluent, mucoid or watery colonies of encapsuled strains of *Str. pyogenes*, i.e. the so-called *Str. epidemicus*, and the more matt, irregularly edged colonies of the chronic mastitis *Streptococcus*.

Haemolysis. In agar plates containing 5 per cent. ox or horse blood sown from a serum-broth culture, growth is indicated at the 18th hour by haemolysed spots of varying size and clearness, though at this time colonies are often not visible to the naked eye. On the second day the colonies are better developed, but even then they are only just visible, and are surrounded by broad zones of clear haemolysis up to 3 or 4 mm. diameter (Figs. 1, 2, 2*a*). At this time the colony zone ratio around the larger colonies is about 1/2 to 1/15. This may be compared with a colony zone ratio of about 1/2 to 1/6 (zone 1–3 mm.) with the haemolytic streptococci of chronic mastitis (Figs. 4, 4*a*), and a ratio of about 1/5 to 1/10 (zone 2–4 mm.) with *Str. pyogenes* (Figs. 3, 3*a*). The colonies of these two groups are nearly always larger than those of the low-acid *Streptococcus*. Surface colonies on ox-blood agar also stand on a broad haemolysed area, and colony development and width of zone are not diminished by adding aesculin and crystal violet in the selective medium recommended by S. J. Edwards(5) for isolating mastitis streptococci. The organism does not split aesculin.

In the tube test with 5 per cent. rabbit red cell suspension the low-acid *Streptococcus* caused haemolysis within an hour and frequently within 10–15 min., whereas the haemolytic streptococci commonly present in mastitis haemolyse slightly if at all under these conditions. A similar difference between the two groups is shown when the haemolytic titre of 24-hour cultures in 1 per cent. serum broth is more accurately determined by adding diminishing amounts of centrifugate to 1 c.c. of 1 per cent. suspension of washed red cells. With the low-acid *Streptococcus* the M.H.D. (within 2 hours at 37° C.) was found to lie between 0·01 and 0·001 c.c., except with nine strains where the M.H.D. was between 0·1 and 0·01 c.c. This titre is similar to that of *Str. pyogenes*, but is notably higher than that of haemolytic mastitis streptococci the titre of which is 1·0 c.c. or over.

A number of cultures of the low-acid *Streptococcus* were tested after 6 months' storage in meat medium in the cold and were found to have retained unchanged their haemolytic power. Two strains were also transferred daily for 20 days in 1 per cent. serum broth, but this caused no change in their haemolytic power or in their microscopic, cultural or fermentation characters.

Biochemical activity. The following substances were tested: glucose, lactose, saccharose, salicin, mannite, inulin, raffinose, glycerine, sorbite and trehalose. Acid production occurred as a rule with glucose, lactose, sorbite and trehalose, while salicin, mannite, inulin and raffinose were invariably negative. Some strains failed to produce acid from lactose when freshly isolated, and acid production was usually absent with saccharose. Many strains were tested

more than once, and where the *p*H is mentioned the lowest value observed is given.

The technique was similar to that given by us in a previous paper(6), but with glycerine, sorbite and trehalose the casein-digest medium described by P. R. Edwards(1) was used. In the case of glucose the final *p*H was 5·0–5·2 with twenty-two strains; 5·3–5·5 with eleven strains, while with one the *p*H did not fall below 5·8. With lactose, one strain gave *p*H 4·9; thirty-two gave a final *p*H 5·0–5·3; with one the *p*H reached 5·9 and with one fermentation was partial (*p*H 6·6). Twenty-four strains were quite negative in glycerine, while two strains brought the *p*H to 6·0. One strain fermented sorbite only slightly (*p*H 6·7) and two strains had only a slight action on trehalose (*p*H 6·4 and 6·6). An outstanding character was the failure to attack saccharose; thirty-one strains had no effect on this sugar, with three the effect was slight or partial (*p*H 6·8, 6·7, 6·4), and only one fermented fully (*p*H 5·2).

All strains produced slight acidification without coagulation in litmus milk, failed to reduce methylene blue in milk containing the dye in a concentration of 1/20,000 and failed to hydrolyse sodium hippurate in the peptone-pepsin medium of Ayers and Rupp(7).

Pathogenicity. Two strains were tested for the production of soluble *toxin* by cultivation for 4 days in 1 per cent. serum broth, but filtrates diluted 1/20 gave no reaction when injected intradermally into a susceptible person.

Twelve strains were tested on mice, each animal being given intraperitoneally 0·5 c.c. 24-hour 1 per cent. broth culture, but all survived for 7 days. Two of the same cultures were tested intravenously in rabbits, a dose of 5·0 c.c. being used for each of two animals, but again without noticeable ill effect.

An Ayrshire cow (No. 1) in full milk was injected in the left hind-quarter of the udder with 3·0 c.c. 24-hour culture in 1 per cent. serum broth of a typical strain. The milk of this cow had been examined in the laboratory a number of times during the previous 6 months, but no abnormalities had been detected. At the time of inoculation, the culture was diluted with 7·0 c.c. milk from the left hind-quarter at the afternoon milking, and immediately milking was finished the inoculum was carefully passed into the sinus through a teat siphon and gently massaged into the gland. Next morning the quarter was only partially emptied, but after that full milking was resumed. The injection caused no physical disturbance in the gland and the milk appeared normal to the naked eye. During the first 4 days there was an increased deposit on centrifuging which reached 1 per cent. by volume, but the organism inoculated could not be recovered from the fore- or mid-milk after the first 24 hours. In the bacteriological test, the deposit from 10 c.c. milk was plated in ox-blood agar containing crystal violet to a concentration of 1/300,000. In these circumstances, with the milk taken on the morning after injection eighty colonies of the low-acid *Streptococcus* developed in the plate, and with milk taken on the same afternoon only eight colonies were present.

Streptococcus infrequens.

A detailed description of this group is unnecessary, since, apart from the effect upon salicin and mannite, the characters were broadly similar to those of the low-acid *Streptococcus*. The organism produced haemolysin as actively as the low-acid *Streptococcus*; in ox-blood agar plates the zone of haemolysis about deep colonies was broad and clear, but the colonies themselves tended to be slightly larger than those of the low-acid *Streptococcus*. Acid was produced from glucose, lactose, salicin, mannite, and irregularly from glycerine, acid production tending to be somewhat greater than with the low-acid *Streptococcus*. Five strains were tested in sorbite and trehalose medium and both substances were fermented. Inulin and raffinose were not fermented, while just over half the strains failed to ferment saccharose. One strain was tested on mice intraperitoneally and on a rabbit intravenously in doses respectively of 0·1 and 5·0 c.c. of a 24-hour serum broth culture without noticeable effect.

Hadley and Frost(8) tested a strain of *Str. infrequens* of human origin on a cow's udder and found that it did not set up mastitis.

An Ayrshire cow (No. 2) in full milk was injected in the left fore-quarter with 5 c.c. 24-hour culture in 1 per cent. serum broth, cultures sown from two strains being mixed for the purpose in equal amounts. The method of injection and the subsequent treatment of the animal was the same as with cow 1 above. The injection caused a slight firm swelling of the gland which persisted for 2 days. The milk of this quarter was not grossly altered in appearance, but for the first 4 days it contained small clots and there was a slightly excessive deposit on centrifuging; the alkalinity of the milk was also abnormal during this period. The streptococci injected could not be cultivated from the milk of the left fore-quarter beyond the first 24 hours and then only in very small numbers.

SEROLOGICAL CHARACTERS.

Low-acid strains were compared with the *Str. infrequens* strains by direct agglutination and agglutinin absorption. Tests were made in the first place on slides at room temperature according to the technique previously described by Stableforth(9). Since the strains gave stable suspensions, the opportunity was taken to confirm the results by tests carried out at 55° C. for 4 hours in tubes in the ordinary way. The suspension used for the slide test contained about 20,000 million organisms per c.c., whereas that for the tube test contained about 1000 million per c.c. The results were similar, but of course agglutinin titres were higher by the tube method.

Preliminary observation showed that the low-acid streptococci and *Str. infrequens* were distinct from any of the serological types of group I mastitis streptococci (*Str. agalactiae*) so far described (Stableforth(9)), as well as from strains of *Str. pyogenes* isolated from the udder (Minett, and Stableforth(6)).

Sera were then prepared in rabbits against two low-acid strains, two strains of *Str. infrequens* and a *Streptococcus* presenting somewhat similar features derived from a human throat. It was found that the low-acid and *Str. infrequens* strains were agglutinated approximately to titre (about 1/30 by the slide test and about 1/160 by the tube test) by sera prepared against both groups of organisms. On the whole, titres were slightly higher with suspensions of the homologous group. Distinction between the low-acid and *Str. infrequens* groups was brought out, however, by absorption methods. For this purpose, serum diluted 1/10 was mixed overnight in the refrigerator with one-quarter of its volume—or in some cases with an equal volume—of a suspension as used in the slide test, the final volume of the mixture being made up with saline where necessary. After centrifuging the absorbed sera were tested against a number of strains by both slide and tube methods. The results showed that low-acid strains removed from both sera all or nearly all the agglutinin for both groups, even with the lower absorbing dose. On the other hand, strains of *Str. infrequens* removed from both sera all the agglutinin against themselves, even with the lower absorbing dose, but they did not reduce by more than one-half the titres of low-acid serum for low-acid strains; in fact, the amount of absorption was not increased by increasing the dose. Sera specific for the low-acid strains could, therefore, be readily obtained by absorption of low-acid sera by *Str. infrequens*. Finally, serological methods failed to show any relationship between the human throat *Streptococcus* and the organisms described in this paper.

Discussion.

The possibility that non-pathogenic haemolytic streptococci may be present in milk does not seem to have received much attention. There are at least two reasons for this. In the first place, media containing blood are used as a rule only by those who are concerned with pathogenic bacteria; secondly, haemolytic streptococci other than pathogenic forms, are not common in milk, and even in the milk of individual cows they never seem to occur in large numbers. In view of the possibility, however, that haemolytic streptococci dangerous to human health may occur in milk, it is important to recognise that there also exist streptococci, which, while similar to pathogenic species in certain respects, possess the somewhat unusual combination of properties of very active haemolysis and lack of pathogenicity.

Brown, Frost and Shaw (3) and Frost, Gumm and Thomas (4), in describing the haemolytic streptococci of milk, outline a procedure for their differentiation, and the first-named authors discuss the relative values of the tests proposed. The final *p*H in 1 per cent. glucose broth and the hippurate test are regarded as the most useful, while virulence tests on mice are considered less reliable. There is a close similarity between the low-acid streptococci described in this paper and the low-acid-producing streptococci described by Jones in 1920 (2), the differences being comparatively unimportant, viz. formation of a soft clot

in litmus milk by Jones's thirteen strains and fermentation of saccharose by all strains save one. Our strains merely acidified litmus milk and generally failed to ferment saccharose. On the other hand, the non-mannite-fermenting *Streptococcus* designated by Frost and his co-workers as *Str. asalignus* appears to be different, since it splits hippurate under conditions in which our strains gave negative results. No mention is made by Frost of the work of Jones. So far as can be judged, the mannite-fermenting streptococci described above correspond to those mentioned by Jones and described by Brown, Frost and Shaw as resembling *Str. infrequens* of Holman's classification. Two-thirds of our strains, however, failed to ferment saccharose and again there was no appreciable splitting of hippurate.

Serologically, it has been shown that the two groups are reciprocally agglutinable, but that, whereas the low-acid strains completely absorb the sera of both groups, strains of *Str. infrequens* only remove the antibodies for themselves. These facts show that the low-acid *Streptococcus* possesses two separate antigens, of which only one is present in *Str. infrequens*.

The possibility that the low-acid *Streptococcus* might be of human origin was considered by Jones, but no evidence was found to support this. In view of the sparse distribution of the organism in udder milk, we too have considered the possibility that it might be a normal inhabitant of the human throat and that its presence in the cow's udder is in a sense accidental. There is, however, nothing to show that this is the explanation. It is true that, as Dr F. Griffith has informed us and as Bliss and Long (10) have recently reported, there are occasionally found in the human throat streptococci which when growing in or on blood agar form minute colonies surrounded by clear haemolytic zones. Through the kindness of Dr Griffith, we have examined four strains of this kind, but, although they were strongly haemolytic in ox-blood agar, they were non-haemolytic in the tube test, with a 5 per cent. suspension of red cells, and they differed also in other ways, *e.g.* in fermenting trehalose.

SUMMARY.

A description is given of the characters of certain streptococci which in small numbers are sometimes found in the milk of individual cows. Although the organisms possessed high haemolytic activity, they showed no evidence of pathogenic power, either for the cow's udder or for ordinary small laboratory animals. Not only are they different from the streptococci of bovine mastitis, but they are also to be distinguished from streptococci of the *Str. pyogenes* group which are occasionally found in milk. One of their principal characters is the formation on solid media of minute colonies, consisting of relatively minute cocci, which grow in broth as very short chains.

The fifty-eight strains studied could be divided into two groups on biochemical grounds: (*a*) those fermenting glucose and lactose with production of small amounts of acid, (*b*) those which ferment mannite and salicin as well. The former are referred to as "low-acid" strains and the latter as *Str. infrequens*,

as they bear a resemblance to the organism given this name in Holman's classification. Fermentation of sorbite and trehalose occurred with streptococci of both groups.

The two groups could be distinguished from *Str. pyogenes* and the ordinary mastitis *Streptococcus* by direct agglutination. For distinguishing the two groups from each other, agglutinin absorption methods were required. It was then found that the low-acid *Streptococcus* possessed two antigens of which one only was represented in *Str. infrequens*.

DESCRIPTION OF PLATE I

Fig. 1. Low acid *Streptococcus*. Primary from milk deposit. Half natural size.
Fig. 2. Low acid *Streptococcus*. From serum broth culture. Natural size.
Fig. 2*a*. Colony from Fig. 2. × 12.
Fig. 3. *Str. pyogenes*. From serum broth. Natural size.
Fig. 3*a*. Colony from Fig. 3. × 12.
Fig. 4. *Str. agalactiae*. From serum broth. Natural size.
Fig. 4*a*. Colony from Fig. 4. × 12.

All 48-hour cultures in crystal violet ox-blood agar.

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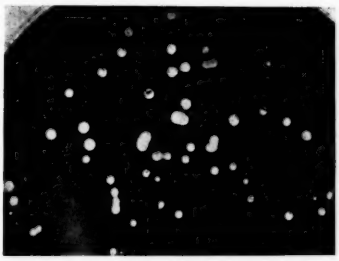

Fig. 1.

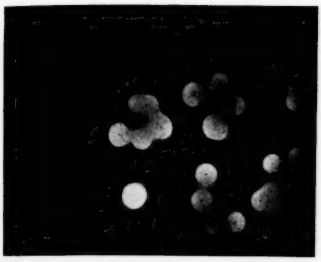

Fig. 2.

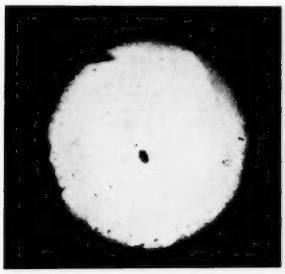

Fig. 2*a*.

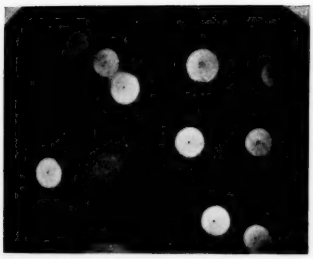

Fig. 3.

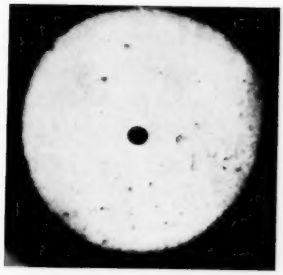

Fig. 3*a*.

Fig. 4.

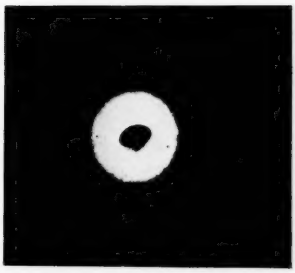

Fig. 4*a*.

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86. A COLIFORM ORGANISM ISOLATED FROM MILK

THE EFFECT OF TEMPERATURE ON THE FERMENTATION OF LACTOSE

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During the cheese-making season of 1932 it was found that certain batches of milk gave very gassy curd in which the development of acidity was unusually slow. The gas production continued even in the moulds, and the cheese were misshapen and eventually putrefied, being quite unfit for use. Each of these batches of milk had been collected during the week-end, so that a portion had been stored at only moderately low temperatures for about 48 hours. The standard plate count of the bulk milk was not abnormally high (*e.g.* 15,000 and 10,400 per 1 ml.), and coliform organisms were present in 1/10 ml. as shown by the usual presumptive technique. Parallel plates which had been made at the same time on beerwort agar and incubated at 20° C. showed, on the other hand, very much higher counts, *e.g.* 875,000 and 182,000 per 1 ml. respectively, indicating the presence of large numbers of organisms which were not detected by means of the ordinary method of plating. The storage temperature was very favourable for the growth of these organisms, and they were found to multiply rapidly in test samples stored under similar conditions.

The original beerwort agar plates appeared to contain almost pure cultures of organisms giving an active gas production in milk at 20° C. Pure cultures of these were obtained by repeated plating on beerwort agar at 20° C. Their morphology and cultural reactions were as follows:

Morphology. Short rods, non-spore-bearing, non-capsulated.

Growth on nutrient agar. At 37° C. very poor; at 20 and 30° C. good, waxy.

Growth on beerwort agar. At 37° C. fair, moist; at 20 and 30° C. abundant, very moist.

Growth of beerwort agar + saccharose. At 20° C. abundant, very moist.

Growth on potato. At 20° C. good, moist.

Growth in Lemco gelatine. At 20° C. slight on surface, good to bottom of stab, slow liquefaction, not evident until about the end of 1 month.

Growth in carbohydrate media. At 37° C. acid and slight gas in dextrose and fructose. Acid in glycerol, arabinose, mannitol, dulcitol, galactose, saccharose, lactose, maltose, raffinose, salicin, mannose. No reaction in adonitol, sorbitol, inositol, inulin. At 30° C. acid and gas in arabinose, mannitol, dextrose, fructose, galactose, saccharose, lactose, maltose, raffinose, salicin, mannose. Acid in glycerol, dulcitol, dextrin. No reaction in adonitol, inulin, sorbitol, inositol.

Reaction in litmus milk. At 37° C. rapid development of acidity with formation of firm curd. No gas production. At 20 and 30° C. development of acidity and vigorous evolution of gas—coagulation—bleaching of litmus—eventual alkaline reaction and complete digestion of curd.

Reaction in bile salt lactose broth. At 37° C. acid; at 20 and 30° C. acid, gas.

Growth in Koser's citrate medium. At 37° C. positive; at 20° C. growth better than at 37° C.

Indole test. At 37° C. negative; at 20° C. negative.

Voges-Proskauer test. At 37° C. positive; at 20 and 30° C. positive.

Methyl-red test. At 37° C. positive; at 20 and 30° C. negative.

Motility. Actively motile, especially at 20° C.

Reaction to Gram stain. Negative.

The organisms were considered to be members of the coli-aerogenes group, and in their motility and liquefaction of gelatine resembled *Bact. cloacae.* They differed from the type strain used for comparison in their greater degree of motility, their very slow rate of liquefaction of gelatine and their preference for vegetable media and relatively low temperatures. Another point of difference was their strong proteolytic action on milk.

An interesting feature of this organism (which has been designated B_2) was the sharp distinction between its fermentation reactions at 30 and 37° C. At temperatures up to and including 30° C. lactose and saccharose were fermented with vigorous expulsion of gas. At 37° C. there was only acid production, gas formation being absent or scarcely perceptible. The behaviour in milk cultures was similar. At 37° C. there was rapid development of acid with coagulation in 5–8 days. At temperatures of 15–30° C. there was a vigorous effervescence of gas within 24 hours together with development of acidity (and bleaching in litmus milk cultures) resulting eventually in a torn curd. When such cultures were stored at room temperature there was a gradual change in the reaction, and by the end of 2 months a definite peptonisation of the casein was apparent. This rapidly progressed until it was complete and the culture consisted of an alkaline liquid. Cultures which had originally been incubated at 37° C. did not, however, undergo this change. Even at the end of 10 months they showed no sign of peptonisation or of loss of acidity. Litmus milk cultures of type strains of *Bact. aerogenes* and *Bact. cloacae* treated in a similar manner also showed no obvious change of reaction or peptonisation at the end of 10 months.

There was a corresponding sharp distinction between the methyl-red test at 37° C. and at lower temperatures. At 37° C. the test was always positive, there being no sign of a reversion of reaction even at the end of 2½ months. At temperatures of 20–30° C. the test was negative, although the reversal was somewhat slower than in parallel cultures of type strains of *Bact. aerogenes* and *Bact. cloacae.* On the other hand, the Voges-Proskauer test was positive, and the indole test negative at all temperatures, indicating that changes in the carbohydrate metabolism were the chief cause of the differences

between the reactions at 30 and 37° C. Similar changes of metabolism were not detected for *Bact. aerogenes* and *Bact. cloacae* (cf. Table I).

Table I.

	B_2		*Bact. cloacae*		*Bact. aerogenes*	
Test	Inc. at 30° C.	Inc. at 37° C.	Inc. at 30° C.	Inc. at 37° C.	Inc. at 30° C.	Inc. at 37° C.
Growth on nutrient agar 48 hours	++ (waxy)	+ (waxy)	+ (waxy)	++ (waxy)	++ (waxy)	+++ (waxy)
Growth on beerwort agar 48 hours	+++ (moist)	++ (moist)	+ (waxy)	++ (moist)	+.(waxy)	++ (waxy)
Reaction in litmus milk 24 hours	A, G	A	Slight A, slight G	Slight A, G	Slight A, G	A, G
8 days	A. G Bleach, torn curd	A Coag.	A, G Slight bleach	A, G Bleach coag.	A, G Slight bleach	A, G Bleach coag.
10 months	Alk. Complete digestion	A Coag.	A Torn curd	A Slightly torn curd	A No coag.	A Coag.
Methyl-red test 24 hours	+	+	−	−	−	−
3 days	+	++	−	−	−	−
6 days	−	++	−	−	−	−
1 month	−	++	−	−	−	−
Voges-Proskauer test 6 days	+	+	+	+	+	+
1 month	+	+	+	+	+	+
Indole test 11 days	−	−	−	−	−	−
Reaction in bile salt lactose broth 48 hours	A, G	A	A, G	A, G	A, G	A, G
Motility 6 hours	+ (active)	+ (in a few cells)	+	+	−	−

+=moderate reaction. ++=good reaction. +++=very good reaction. A=acid production. G=gas production.

The occurrence of organisms such as that described, which, although in reality an active lactose fermenter, would not be recognised as such by means of the ordinary routine tests, is of importance in the examination of milk. There is no evidence to show that it was of faecal origin, in fact their preference for relatively low temperatures and vegetable media points to the soil or plants as its habitat. Its significance in milk which is destined for immediate consumption may not, perhaps, be great, but it would be a considerable factor in the deterioration of milk products especially butter and cheese. Similar, though not identical, organisms have been described by Grimes and Hennerty (1) who isolated them from butter, in which apparently they may readily be found. This raises the question of the wisdom of the universal use of a temperature of 37° C. in the test for organisms of the coli-aerogenes group. In any event, tests at lower temperatures should be included for milk which is to be made into butter or cheese.

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637.143:532.73

87. FACTORS AFFECTING THE SOLUBILITY OF MILK POWDERS

III. SOME PHYSICO-CHEMICAL PROPERTIES OF CONCENTRATED SOLUTIONS OF MILK SOLIDS

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From the Hannah Dairy Research Institute, Kirkhill, Ayr.

(With 4 Figures.)

In the first paper of this series (Wright (1)) it was shown that the proteins of milk, and in particular the casein, were very readily coagulated when highly concentrated solutions of milk solids were heated. Thus, while it took upwards of 24 hours' heating at 100° C. to induce coagulation in a sample of reconstituted separated milk of normal solids content (*circa* 9 per cent.), coagulation took place in solutions containing 40–50 per cent. milk solids within a few minutes. In still more concentrated solutions, containing over 60 per cent. milk solids, the proteins were rendered insoluble by heating at 100° C. for only a few seconds.

Among the factors causing this marked decrease in protein stability it seemed likely that the increasing concentration of electrolytes in the highly concentrated solutions of milk solids would be of outstanding importance. It therefore appeared desirable to make a detailed study of those physico-chemical properties of concentrated milk solutions which are primarily dependent on ionic concentration. The properties investigated were the electrical conductivity, the hydrogen-ion concentration, and (as a means of measuring the general osmotic activity of the solutions) the depression of the freezing-point.

Technique.

(*a*) *Preparation of solutions.*

In order to obtain comparable results throughout the entire series of experiments, all solutions of milk solids were made up from the same highly soluble brand of spray-dried separated milk powder. This powder was similar to that used by Wright *loc. cit.* and was readily and completely soluble in cold water. The method employed in the preparation of the solutions, which were invariably made up in duplicate, was as follows: The requisite quantity of milk powder was weighed into a Pyrex boiling tube, a few millilitres of water were added, and the mixture was stirred with a steel rod into a thick paste. After further dilution the contents of the tube were transferred to a 50 ml. standard

flask, and made up to the mark with washings. A drop of caprylic alcohol was added to prevent frothing. This was essential in the highly concentrated solutions where the occlusion of small air bubbles was otherwise liable to lead to serious inaccuracies. By this method homogeneous solutions could be readily prepared up to the highest concentration used in the experiments, *i.e.* 65 per cent.[1] Although the viscosities of the solutions increased markedly with the higher concentrations of milk solids, all the solutions were definitely fluid and could be poured from one vessel to another.

Conductivity measurements were made very shortly after preparation of the solutions (within 2 hours); *p*H and freezing-point determinations were made after standing for 20–24 hours at 3–4° C.

It may be noted here that when solutions containing 34 per cent. or more of milk solids were allowed to stand overnight, a white sediment invariably settled out. On investigation this sediment proved to be lactose. The significance of this fact is dealt with in a later section.

(*b*) *Physico-chemical measurements.*

Electrical conductivity. Conductivity measurements were made in a cell consisting of two vertical platinum electrodes, each about 1 cm. square, rigidly sealed into glass tubes, the upper ends of the latter being firmly fitted into the ebonite lid of the cell. The electrodes were heavily coated with platinum black. The readings were made on an Electrolytic 10-metre Bridge Wire (Cambridge Instrument Co., Ltd.) in conjunction with a buzzer and telephone. All measurements were made at 18° C. The cell constant was checked each day, but remained constant at 0·112.

Freezing-point determinations. These were made with a Hortvet cryoscope, fitted with a Beckmann thermometer. Check determinations on distilled water showed a variation of only 0·01° C. throughout the course of the work. In the solutions of low milk solids concentrations considerable supercooling was experienced; for instance, in the 17·8 per cent. solution the degree of supercooling was 1·72° C. While this value greatly exceeds the optimum suggested by Findlay (2), two successive readings never showed a greater difference than 0·005° C., and in general gave identical values. In solutions containing more than 40 per cent. milk solids the degree of supercooling was smaller, and it continued to decrease up to 65 per cent. milk solids content. At this concentration the degree of supercooling was about 0·30° C.

Hydrogen-ion concentrations. Determinations of the hydrogen-ion concentration were made with the quinhydrone electrode. In the highly concentrated solutions there was always a slight drift, but even with the highest concentration (65 per cent. milk solids) this only amounted to 1·0 millivolt in 5 min. A drift of this magnitude would not appreciably alter the results. According to Clark (3) such drifts in complex solutions are due to secondary

[1] Throughout this paper percentage concentrations are expressed as *g. solute per* 100 *g. water* and not as g. solute per 100 ml. solution.

reactions after normal equilibrium has been attained. In order to minimise any small errors arising from these drifts, measurements were always made after the solution had been in contact with the quinhydrone for the same period of time (10 min.), while check readings were made 5 min. later.

Discussion of results.

The results are shown in Figs. 1–3. They may be conveniently discussed under three heads:

(*a*) *Electrical conductivity* (Fig. 1).

The conductivity of a 10 per cent. solution of milk solids, corresponding to normal separated milk, is shown from Fig. 1 to be $40{\cdot}0 \times 10^{-4}$ mhos. This value

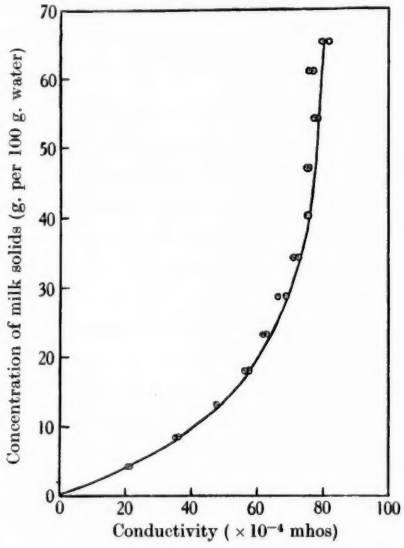

Fig. 1.

falls outwith the general range noted by Rogers(4) for whole milks, *i.e.* 45 to 48×10^{-4} mhos. Rogers' figures appear, however, to be based on determinations made at 25° C., whereas in the present work the temperature employed was 18° C. It was found that an independent measurement made on the 10 per cent. solution at 25° C. gave a conductivity of $49{\cdot}5 \times 10^{-4}$ mhos.

From the general shape of the curve shown in Fig. 1, it will be seen that the relation between conductivity and concentration of milk solids is not linear, but that there is a definite retardation in the rate of increase of conductivity with increasing concentration of milk solids. At high concentrations this retardation becomes so marked that, beyond 40 per cent. milk solids, the increase in conductivity is scarcely appreciable.

Two main factors might be expected to contribute to this result, namely, the degree of ionisation of the electrolytes present, and the increasing viscosity of the solutions. As regards degree of ionisation, it is well recognised that the conductivity of milk is chiefly due to the mineral salts, the effect of the calcium caseinate and colloidal calcium phosphate present being negligible in comparison with that of the highly ionised constituents. Since the degree of ionisation of electrolytes normally decreases with increasing concentration, it would be anticipated that the conductivity of a highly concentrated solution of milk solids would be (relatively) lower than that of a more dilute solution. This ionisation effect could not, however, be of sufficient magnitude to account for the very marked retardation of conductivity in solutions containing more than, say, 18 per cent. milk solids. It may be assumed, therefore, that the marked retardation in conductivity with increasing concentration is chiefly attributable to the second factor, namely, the increasing viscosity of the highly concentrated solutions, which will reduce the mobility of the constituent ions. Bateman and Sharp(5) have shown that the absolute viscosity of normal skim milk approximates 1·5, while that of evaporated skim milk (containing 25 per cent. milk solids) is about five times this value. Moreover, Greenbank, Steinbarger, Deysher and Holm(6) found that, at concentrations of 45–50 per cent. solids, skim milk exhibited the properties of plastic flow. The fact that the conductivity approaches a maximum in such highly viscous or plastic solutions can be readily understood.

(*b*) *Freezing-point depression* (Fig. 2).

The curve showing the relation between concentration of milk solids and the depression of the freezing-point consists of two distinct parts, the first extending from 0 to 34 per cent. of milk solids, and the second from 34 per cent. upwards. The sharpness of the break in the curve at 34 per cent. indicates that at this point there is a definite physical change in some component of the system.

Dealing first with the break at 34 per cent. of milk solids, it has already been noted (p. 237) that, above this concentration, a white sediment was invariably deposited from the solutions on standing overnight. On analysis this sediment was found to consist of pure lactose. It therefore appeared possible that a concentration of 34 per cent. milk solids represented the maximum solubility of lactose at 0° C., and that the increase in freezing-point depression beyond 34 per cent. was due to the remaining osmotically active milk constituents, chiefly to dissolved electrolytes. Two procedures were adopted in order to test this theory.

In the first place determinations were made of the amount of soluble lactose present in varying concentrations of milk solids. Solutions containing varying quantities of milk solids were prepared as previously described, and were left in an ice chest at 3° C. overnight. They were then removed from the ice chest and immediately centrifuged. Determinations of the soluble lactose

present in the upper layer were made by the volumetric method of Shaffer and Hartmann (7), after removal of the protein by precipitation with 10 per cent. phosphotungstic acid. The results, which are shown in Fig. 3, demonstrate clearly that the maximum solubility of the lactose in solutions of milk solids is reached at a concentration at approximately 34 per cent. of the latter, and that beyond this point the solubility remains (within experimental error) constant.

In the second place, since the theoretical freezing-point depression due to the dissolved lactose can be calculated from its molecular weight, it is possible to determine the expected freezing-point depression due to other milk constituents. This has been done, and the dotted line XY shows the direction of the curve which would be expected if the increase in freezing-point depression

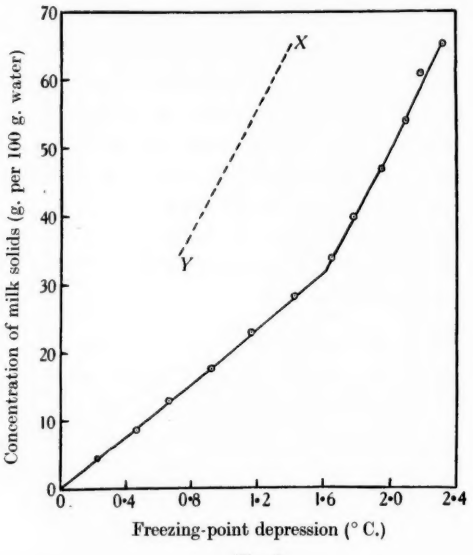

Fig. 2.

beyond 34 per cent. were due to milk solids other than lactose. It is obvious that, over the entire length of the curve, the direction of the observed results coincides closely with that of the calculated results.

Turning now to the general shape of the two portions of the observed curve, it will be seen that there is an approximately straight line relationship between concentration of milk solids and freezing-point depression. The very slight curvature shown in both portions of the curve is probably attributable to a small decrease in the ionisation of electrolytes at the higher concentrations of milk solids.

Several workers, notably Newton and Gortner (8), Newton and Martin (9) and Briggs (10), have reported that in solutions of some lyophilic colloids, especially proteins, a definite fraction of the water is "bound" by the colloid, "bound"

water being defined as water which is no longer capable of acting as a normal solvent. It is obvious that, if any considerable quantity of "bound" water existed in the higher concentrations of milk solids (containing up to 20 per cent. of protein) it might exert a considerable influence on the physical properites of the solutions, since it would result in the production of abnormally high concentrations of electrolytes. Such increases in concentration of electrolytes (and of other osmotically active constituents of the system) would, however, be reflected in abnormally high osmotic pressures, and hence in corresponding increases in the freezing-point depression. The linear relationship between concentration of milk solids and freezing-point depression shown in Fig. 2 indicates that "bound" water is absent from the solutions studied.

As confirmatory evidence on this point seemed desirable, the following experiment was carried out. A 65 per cent. solution of milk solids was prepared (the exact quantity of water present being known) and the depression

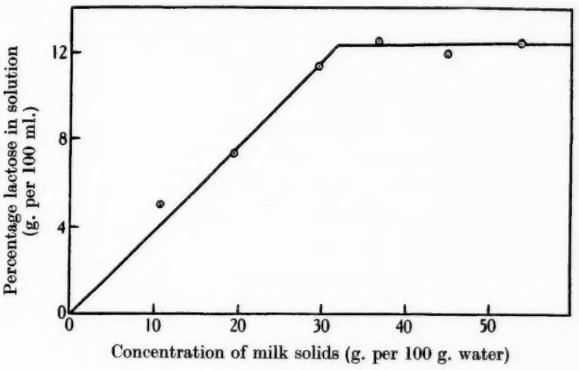

Fig. 3.

of the freezing-point was determined. Sufficient glucose was then dissolved in an aliquot part of this solution to give, with the amount of water present in the aliquot, a 5 per cent. glucose solution. The depression of the freezing-point of this solution was also determined. A further determination of the freezing-point depression was made on a solution containing 5 g. glucose dissolved in 100 ml. pure water. The results were as follows:

	°C.
Freezing-point depression of 65 per cent. milk solids solution containing 5 per cent. glucose	2·870
Freezing-point depression of 65 per cent. milk solids solution alone	2·313
Difference due to dissolved glucose	0·557
Freezing-point depression of 5 per cent. glucose in pure water (duplicates)	0·558 0·555

This result confirms the previous conclusion that, up to concentrations of 65 per cent. milk solids, there is no evidence of the existence of "bound" water.

(c) *Hydrogen-ion concentration* (Fig. 4).

In order to determine whether a direct relationship exists between the concentration of milk solids and the hydrogen-ion concentration, the latter has been plotted as such, and not in *p*H units. It will be seen that the relationship is roughly linear, any increase in the concentration of milk solids being accompanied by an equivalent increase in the acidity of the solution. The slight deviation from a straight line relationship is again explainable on the basis of small differences in the degree of ionisation of the electrolytes present.

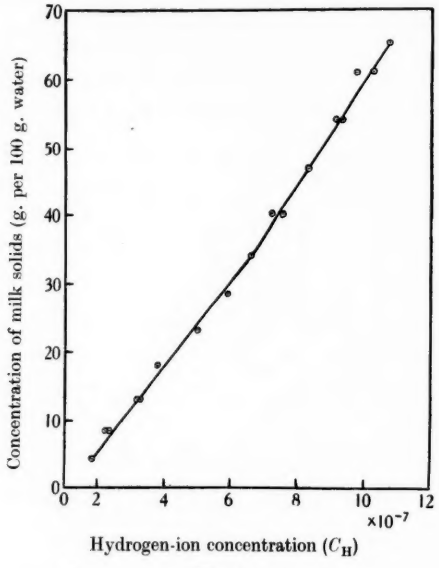

Fig. 4.

APPLICATION OF THE RESULTS TO THE HEAT COAGULATION OF EVAPORATED AND DRIED MILKS.

The primary object of the present work was to ascertain how far alterations in the ionisation and concentration of electrolytes might be responsible for the decreased heat stability of the proteins in high concentrations of milk solids.

As regards determinations of electrical conductivity, these failed to furnish any useful information, owing to the effect of viscosity on ionic mobility in the more highly concentrated solutions. In the *p*H determinations, where this factor was no longer operative, results were obtained which showed that a definite increase in hydrogen-ion concentration took place with increasing concentrations of milk solids. Thus, while a 10 per cent. solution (roughly equivalent to normal skim milk) had a *p*H of about 6·6, the *p*H of a 30 per cent. solution was 6·20, while that of a 65 per cent. solution approximated 5·97. The

sensitivity of casein to heat coagulation increases towards its isoelectric point (*p*H 4·6–4·7), and the present writers have, in fact, found that milk solutions of normal solids content may be very rapidly coagulated by heating at 120° C. when the *p*H is on the acid side of 6·0. It will be seen, therefore, that the concentration of milk solutions (such as takes place in commercial evaporation or drying), by lowering the *p*H, will inevitably produce conditions favourable to heat coagulation. In this connection it is interesting to note that Fouassier (11) suggested over 10 years ago that the protein insolubility induced by storing milk powders in a moist condition was due to the high concentration of the "natural acidity" of the fresh milk in the slightly moist powder.

Turning now to the freezing-point data it has been shown that, throughout the range of concentrations studied, the observed depressions of the freezing-point coincide closely with the calculated values, provided that allowance is made for the limited solubility of lactose at 0° C. It is reasonable to assume that a similar linear relationship will hold for the elevation of the boiling-point. Moreover, since, at the boiling-point, complete saturation of solutions of milk solids with lactose cannot be reached, there will be no break in the boiling-point curve equivalent to that which is found at a concentration of 34 per cent. in the freezing-point curve. Scott (12) has, in fact, found that, in solutions of milk solids up to 50 per cent. and at temperatures between 140 and 150° F., the relationship between concentration and boiling-point elevation is linear. In view of the fact that, in commercial evaporative processes such as roller-drying, the temperature to which the heated milk is subjected is dependent on its boiling-point, the establishment of a basis on which the latter value can be calculated is of definite practical importance.

Summary.

1. The effect of increasing concentrations of milk solids on the degree of ionisation of electrolytes cannot be measured by determinations of electrical conductivity, owing to the influence of viscosity on ionic mobility.

2. An approximately linear relationship exists between concentration of milk solids and the freezing-point depression. Above a certain concentration (equivalent to 34 g. milk solids per 100 g. of water), however, the solutions are saturated with respect to lactose, and any increase in freezing-point depression beyond this point is limited to that due to the other osmotically active constituents of milk.

3. There is no evidence of the existence of "bound" water in solution of milk solids up to concentrations equivalent to 65 g. milk solids per 100 g. water.

4. An approximately linear relationship exists between concentration of milk solids and hydrogen-ion concentration, increase in the concentration of milk solids resulting in a markedly increased acidity.

5. The applications of these results to the heat coagulation of evaporated and dried milks are briefly discussed.

ACKNOWLEDGMENTS.

The three papers in this series form part of a co-operative investigation into certain scientific aspects of the condensing and drying of milk which is being carried out in conjunction with the Royal Technical College, Glasgow. The authors desire to record their indebtedness to Prof. A. L. Mellanby and to Dr A. W. Scott for their advice and assistance throughout this investigation. They also desire to express their thanks to Mr J. B. Pettigrew, B.Sc., for his assistance in the earlier part of the present work. One of the authors (G. R. H.) is indebted to the Agricultural Research Council for the award of a Research Scholarship during the tenure of which this work was carried out.

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612.664.12

88. A TECHNIQUE FOR THE DETERMINATION OF LACTOSE IN MILK BY THE HAGEDORN-JENSEN METHOD

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(With 1 Figure.)

Iodometric methods have been applied to the determination of lactose in milk by Kolthoff (1), who used freshly precipitated zinc ferrocyanide as protein precipitant, and by Fine (2) who made use of colloidal iron. The reduction of ferricyanide by reducing sugars has been used as a semi-micro method by Issekutz and von Both (3) and has been applied to the estimation of lactose in milk by Gohr (4) who also used ferrocyanide as protein precipitant.

The method of Hagedorn and Jensen (5) has also been widely applied to the estimation of reducing sugars in blood. In speed and accuracy it has many advantages and its scope may be extended to include a wide range of concentrations. Briefly, the method depends upon the reduction of potassium ferricyanide to ferrocyanide when heated in alkaline solution with reducing sugars. The ferrocyanide so formed is then precipitated as the potassium zinc salt and the residual ferricyanide may be estimated iodometrically. The application of this method to the determination of large quantities of reducing sugars—glucose and maltose—has been worked out by Hanes (6), who points out an important advantage over copper methods (Shaffer and Hartmann (7)) in that variations in the amount of dissolved oxygen in sugar solutions do not affect the reducing values obtained by the Hagedorn-Jensen technique. In the present study of the determination of lactose it was desired to standardise the Hagedorn-Jensen ferricyanide method up to concentrations of 5 mg. of lactose per determination and to test its applicability to milk filtrates.

Experimental.

All chemicals used were "Analytical Reagent" quality supplied by the British Drug Houses, Ltd.

Solutions.

A. Potassium ferricyanide ... ... 8·25 g.
Anhydrous sodium carbonate ... ... 10·6 g.

Make up to 1 litre with distilled water and store in an amber glass bottle. This solution should be kept for 2–3 days before use.

B. Potassium iodide ... ... 25 g.
Zinc sulphate ... ... 50 g.
Sodium chloride ... ... 250 g.

Make up to 1 litre with distilled water. Traces of free iodine should be removed from this solution before use by filtration through several thicknesses of filter paper.

C. Glacial acetic acid ... ... 50 ml.

Dilute to 1 litre with distilled water.

D. Soluble starch ... ... 1 g.

Rub the starch to a thin paste with cold water and wash into 50 ml. of boiling water. Boil for 3 min., add 20 g. of sodium chloride, heat gently for 1 min., cool and dilute to 100 ml.

E. 0·01*N* sodium thiosulphate.

Make up by dilution of "ripe," approximately 0·1 *N*/sodium thiosulphate with boiled-out distilled water, adding 10 ml. of pure amyl alcohol per litre. "Ripe" sodium thiosulphate is obtained by storing an approximately 0·1*N* solution of the crystalline salt in amber bottles for several months. Dilutions of such a stock solution keep extremely well and need only be standardised at intervals after the first week. It is convenient to store the 0·01 *N* thiosulphate solution in amber bottles attached to 10 ml. automatic zero burettes graduated in 0·05 ml. divisions and protected by soda-lime tubes.

Standardisation of 0·01*N thiosulphate.*

Measure 5 ml. of a 0·8 per cent. solution of pure potassium iodate into a boiling tube, add 5 ml. of a 2 per cent. filtered solution of potassium iodide and 3 ml. of 5 per cent. acetic acid. Titrate the liberated iodine with the thiosulphate solution, adding two drops of starch solution just before the end-point is reached and proceeding until the blue colour of the starch-iodide complex disappears on shaking.

Determination of reducing power.

Measure 5 ml. of solution A into a Pyrex boiling-tube. Add 5 ml. of a solution containing lactose (range 0·2–5 mg. of lactose per 5 ml. of solution) and heat in a boiling water-bath for 15 min. Reduction of ferricyanide takes place. Cool the tubes in running water for 3 min. It is important that the heating and cooling should be as uniform as possible. Add 5 ml. of solution B and 3 ml. of solution C. A white flocculent precipitate is formed and iodine is liberated. The free iodine should be titrated as described under the standardisation of thiosulphate after the tubes have remained closely stoppered for 4 min. There should be no recurrence of blue colour within 10 min. A running end-point is due to the addition of starch solution while there is still a considerable amount of iodine in the solution. The appearance of a pink colour towards the end-point indicates that the starch solution is too old and should be discarded. It is important to test all solutions with starch at frequent intervals, and discard any which produce a colouration. When these precautions are observed concordant results are readily obtained.

The above procedure should be repeated using 5 ml. of distilled water instead of lactose solution, and the thiosulphate value of the lactose test should be subtracted from that of the blank. This figure is the 0·01 thiosulphate value of the ferricyanide equivalent to the reducing power of the lactose solution. In practice the water blank should be determined at the beginning and end of each series of determinations. Six or ten tubes may conveniently form a series and two series may readily be carried out in one hour.

The standardisation graph for lactose.

A sample of pure lactose monohydrate was dried *in vacuo* over concentrated sulphuric acid for 6 hours. Stock solutions were made such that accurate dilutions could be obtained containing from 0·25 to 6 mg. of lactose in 5 ml. of solution. The reducing power of each solution was determined in terms of 0·01 thiosulphate equivalents. The data are given in Table I and Fig. 1 and the

Table I. *Blanks, ml.* 0·01 N *thiosulphate* 12·32, 12·32, 12·34, 12·34, 12·31, 12·34, 12·28, 12·30.

Mg. of lactose in 5 ml. of solution		0·25	0·50	0·75	1·00	1·25	1·50	2·00	2·25	2·40	3·00	3·30	4·00	4·50
Thiosulphate equivalent of ferricyanide reduced as ml. 0·01 *N*	(1)	0·58	1·10	1·69	2·34	2·83	3·36	4·48	4·92	5·45	6·63	7·03	8·54	9·33
	(2)	0·58	1·16	1·69	2·26	2·83	3·38	4·50	4·94	5·47	6·63	7·05	8·60	9·29
Mean		0·58	1·13	1·69	2·30	2·83	3·37	4·49	4·93	5·46	6·63	7·04	8·57	9·31

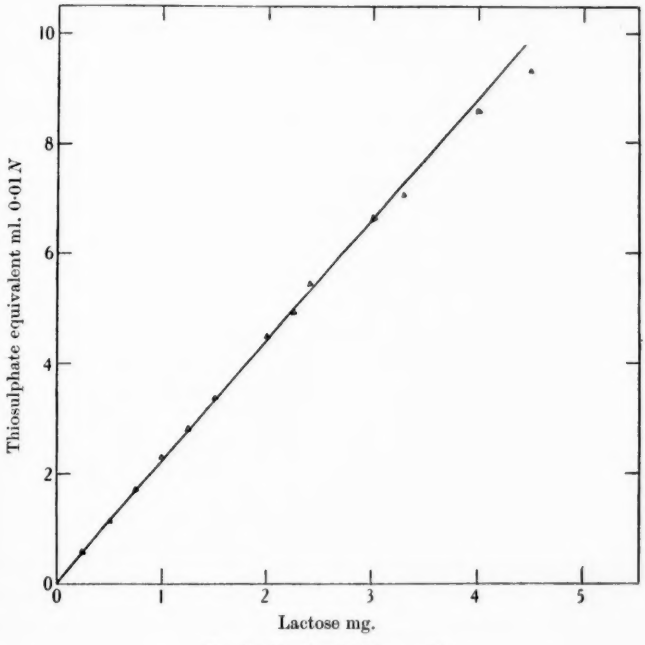

Fig. 1. Standardisation graph.

relationship is seen to be linear within the range adopted. 1 ml. of 0·01*N* thiosulphate is equivalent to 0·453 mg. of lactose monohydrate or 0·430 mg. of anhydrous lactose. This factor may conveniently be used for the conversion of reduction values into lactose. The two figures given in Table I for each concentration of lactose have been derived from different sugar solutions so that the reliability of the procedure is evident.

Application to milk.

A 1 in 100 protein-free dilution of milk has a lactose concentration such that 5 ml. may be employed for its determination by the method given. The protein-free filtrate may be prepared either by the Folin-Wu tungstic acid method or by precipitation with the cadmium sulphate reagent described for blood by Fujita and Iwatake (8). The Folin-Wu precipitation is well known and need not be described. The cadmium sulphate reagent has the following composition:

Cadmium sulphate	13 g.
N sulphuric acid	63·5 ml.
Distilled water to 1 litre.	

One volume of milk is pipetted into 8 volumes of the cadmium sulphate solution and 1 volume of 1·1 *N* caustic soda is added. The solution is filtered after thorough shaking.

In each case the original filtrate may be diluted ten times and 5 ml. taken for each lactose determination by the method now given.

Comparison with the Shaffer and Hartmann cuprous titration procedure.

A protein-free filtrate of milk was made by each of the methods cited and lactose determined by the Shaffer-Hartmann and ferricyanide procedures. In Table II the results of each method are shown. It is seen that there is close agreement between the two methods of determination and also between the two protein precipitation procedures.

Table II. *g. lactose in* 100 *ml. milk.*

	Shaffer-Hartmann method	Hagedorn-Jensen ferricyanide method
Cadmium filtrate	4·744	4·82
Folin-Wu filtrate	4·784	4·80

Conclusion.

The application of the Hagedorn-Jensen ferricyanide reduction procedure to the estimation of lactose in pure solution and in milk is discussed. Results obtained by this method agree closely with the Shaffer-Hartmann macro-cuprous titration method.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION E. THE NUTRITIONAL VALUE OF MILK AND MILK PRODUCTS

Introduction.

General.

Cow's milk:

I. Nutritional value as estimated in laboratory experiments.
  A. Raw milk.
    (*a*) Vitamins.
      (i) Vitamin A.
      (ii) Vitamin B.
      (iii) Vitamin C.
      (iv) Vitamin D.
    (*b*) Other factors.
  B. Treated milk.
    (*a*) Effect of heat.
      (i) General.
      (ii) Vitamins.
      (iii) Other factors.
    (*b*) Irradiated, vitaminised and mineralised milk.
  C. Milk products.
    Butter.
      Vitamin A.
      Vitamin D.

II. The nutritional value of milk for human beings.
  A. Raw milk.
  B. Treated milk.
    (*a*) Pasteurised milk.
    (*b*) Irradiated and vitaminised milk.

INTRODUCTION.

As it is impossible to deal adequately in the available space with the very large number of papers published within the last few years on the various aspects of the nutritional value of milk, it is proposed to deal only with the publications on liquid cow's milk (raw and heated) and on butter. Other milk products, and the milk of animals other than the cow, will be considered in a subsequent review. Most of the papers have been consulted in the original. Where this was impossible and abstract journals had to be used, the references are marked with an asterisk in the bibliography.

GENERAL.

Several papers dealing with the general aspects of the nutritive value of milk have been published during the period covered by this review. Amongst these are the revised edition of a *Bulletin* originally published in 1919 by the Connecticut Agricultural Experiment Station(1), and a popular review by Mitchell(2). Much

useful information will be found in the latest edition of the Medical Research Council's monograph on *Vitamins* (3). Two reports by Hiscock (4, 5) should also be consulted, as well as the extensive review of the nutritional aspects of milk by the White House Conference on Child Health and Protection (6).

The study of relevant literature shows that during the past two years, two problems have been given special attention; (1) the effect of heat treatment on the nutritive value of milk, and (2) the problem of increasing, either by suitable feeding of the cow, or by direct addition, the concentration of factors in which milk is more or less deficient (*e.g.* vitamin D) or which undergo seasonal variations (*e.g.* vitamin A).

COW'S MILK.

I. Nutritional value as estimated in laboratory experiments.

A. *Raw milk.*

(*a*) *Vitamins.*

(i) *Vitamin A.* Only a few papers have been published on the vitamin A content of milk, and as most of the work has been done on butter it is reviewed in the section dealing with this product. MacLeod, Brodie and Macloon (7) have found that, when cows are stall fed throughout the year and receive a uniform ration, the vitamin A content of their milk is remarkably constant. Davis and Hathaway (8) have compared the vitamin A content of the milk of Holstein, Ayrshire, Jersey and Guernsey cows kept under identical conditions of feeding, and found no difference between these breeds. Dann (9) has made the very important observation that the vitamin A concentration in colostrum may be from 10 to 100 times greater than in the later milk from the same cow, independently of season. The significance of this finding, especially in view of the poor permeability of the placenta for vitamin A, is obvious.

(ii) *Vitamin B.* It is now generally accepted that the vitamin B complex may be formed in the rumen of the cow by bacterial synthesis, and it is not surprising to find that the concentration of the vitamin B complex in milk remains more or less constant throughout the year. Gunderson and Steenbock (10) found that neither breed, season nor feeding affect the content of vitamin B_1 in milk. Hess, Light, Frey and Gross (11) noticed that the feeding to cows of 300 g. daily of a yeast rich in vitamin B_1 did not affect the level of this factor in the milk. Krauss and Hunt (12) report similar findings, while Kieferle, Dusch and Hindemith (13) found no increase in the vitamin B complex content of milk after feeding to cows 1·5 kg. of yeast daily. On the other hand, Tscherkes and Jurist (14) have observed an increase of vitamin B_1 in milk after feeding silage to cows on winter rations. Their experimental technique is, however, open to criticism. MacLeod *et al.* (7) found no seasonal variation in either the vitamin B_1 or B_2 content of the milk of their stall-fed cows, but Hunt and Krauss (15), while finding no seasonal variation in vitamin B_1, noted an increase in the vitamin B_2 content of milk when cows had access to pasture. Krauss and Hunt (12) also found that the feeding of yeast to cows increased slightly the level of vitamin B_2, though Krauss and Hayden (16) are inclined not to attach too much importance to this finding. In the same paper, the latter authors determined the effect of wide variations in the protein content of the diet on the vitamin B complex content of the milk, and found that high-protein milk was slightly more efficient than low-protein milk as a source of this factor. In work carried out at the National Institute for Research in Dairying (17, 18), of which only preliminary reports have been published, it was found that in the course of two consecutive years the season and nutrition of the cow had no noticeable effect on the vitamin B complex content of milk. The vitamin B_1 and B_2 content of milk has also been studied by Samuels and Koch (19).

A notable recent development has been the discovery by Kuhn and his collaborators(20, 21, 22) that lacto-flavin, the water-soluble pigment of milk, has a marked vitamin B_2 activity, and is in all probability the vitamin itself. The pigment is also identical with ovo-flavin, the pigment of egg albumin, and is active for rats in doses of 0·005 mg. daily. Other accessory factors of a vitamin nature are also doubtless present in milk, and the work of Stiebeling(23) is representative of the evidence brought forward for the presence in milk of the less well-defined members of the vitamin B complex.

(iii) *Vitamin C.* The vitamin C content of milk is rather low and its biological estimation is therefore somewhat difficult. Kieferle and Eisenreich(24) have found that winter milk contained more vitamin C than summer milk produced on the same farm. Interesting developments have followed the application of the chemical test for vitamin C to milk (making use of the Tillmans reagent, 2, 6-dichlorophenol-indophenol). For example, it has been noted by Schlemmer, Bleyer and Cahnmann(25) that the vitamin C content of milk as measured by this method is subject to marked and irregular variations, and similar day to day fluctuations have been observed by Kon(26). It has since been found by A. T. R. Mattick and Kon(27) that visible light causes the rapid disappearance of the reducing substance in milk, and it would appear that the variations previously observed were caused by differences in exposure to light of the samples. Martini(28) noticed that light had a marked effect on the oxidation-reduction potential of milk. He believed at first that glutathione was the chief factor in this reaction. Later(29), however, he stated that probably ascorbic acid was the cause of the reaction. It is possible that by the action of light vitamin C is not irrecoverably destroyed, but that it undergoes reversible oxidation. It has been pointed out by Tillmans(30) that vitamin C may undergo reversible oxidation by the action of indophenols, hydrogen peroxide or iodine.

(iv) *Vitamin D.* The paucity of information on the vitamin D content of natural milk in the period under review contrasts strangely with the voluminous literature dealing with methods of raising the content of this factor by special feeding of the cow, or by direct addition to the milk. In general, the vitamin D content of natural milk has been estimated in the butter churned from it, as the feeding of whole milk complicates the estimation by altering the Ca/P ratio of the rachitogenic diet.

The *Annual Report* for 1932 of the Director of the South Dakota Agricultural Experiment Station(31) mentions that rats receiving 50 ml. daily of milk produced in direct sunlight "in a dry lot" made better gains than rats similarly fed but receiving milk produced in the absence of sunlight. Rickets was absent from both groups.

(*b*) *Other factors.*

It is now generally agreed that the anaemia caused in experimental animals by the feeding of an exclusive milk diet is due to a deficiency of iron and copper, and that these two metals alone have the power of restoring a normal blood picture. The recent careful work of Krauss(32), and of Orten, Underhill and Lewis(33), bears out in this respect the original findings of the Wisconsin workers. Mitchell(34) was able to cure anaemia in rats on milk diets by the addition of iron alone. The addition of copper merely accelerated recovery in his case and gave higher final haemoglobin levels. It is possible that the work of Skinner, Steenbock and Peterson(35) might help to explain this apparent discrepancy. Schiff, Eliasberg and Joffe(36) believe that copper-free lactose can replace copper in the cure of anaemia. While producing anaemia on an exclusive diet of cow's milk they were unable to bring about the condition by the administration of human milk, probably, according to the authors, owing to its richness in lactose.

In contrast with other workers on the subject, Scott and Erf(37) report that milk

from properly fed cattle is apparently a complete food, and when fed exclusively brings about normal growth and a normal blood picture in the albino rat. They found that the food of the cow has a very marked influence on the anti-anaemic properties of milk, winter feed milk being valueless in this respect. Heavy milkers though properly fed also produced inferior milk. These observations stand so far isolated. Krauss(38) found that the rate of development of anaemia was independent of the season and unaffected by change from dry feed to blue grass pasture. The iron and copper content of the milk was also quite constant. Krauss, Erb and Washburn(39) repeated the experiments of Scott and Erf but failed to confirm their findings. Though Underhill, Orten, Mugrage and Lewis(40) report that an exclusive milk-iron-copper diet will maintain rats in an apparently normal condition for prolonged periods of time, it appears from the recent work of the Wisconsin school that iron and copper are not the only minerals in which milk is deficient. While some of their earlier claims as to the action of manganese when added to a milk-iron-copper diet have not been substantiated by later work (Skinner, Van Donk and Steenbock(41)), it would appear at present that when a milk-iron-copper diet is supplemented with manganese, growth is enhanced (Skinner, Peterson and Steenbock(42)), chiefly owing to stimulation of appetite. On the other hand the addition of energy in the form of manganese-free sucrose to a milk-iron-copper diet also markedly improves growth and brings about, in female rats, resumption of an almost normal ovulatory rhythm which is deranged on the milk-iron-copper diet (Van Donk, Steenbock and Hart(43)). The rôle of manganese in this respect is therefore not quite clear, but its essential nature in nutrition seems demonstrated by experiments of Orent and McCollum(44) who used synthetic manganese-free diets. Kemmerer, Elvehjem, Hart and Fargo(45) have reported exceedingly rapid growth of rats and pigs receiving an exclusive diet of milk supplemented with iron, copper and manganese, and also, in the case of pigs, with cod-liver oil. The addition of sucrose instead of manganese has not, however, been tried by these authors. The problem has quite recently been studied by Keil, Keil and Nelson(46). These authors noticed in their rats a marked improvement in growth and a temporary improvement in reproduction when manganese was added to a milk-iron-copper diet. They also tried adding other minerals, but were unable to carry reproduction beyond the fifth generation. They believe that the deficiencies of a whole milk diet in this respect are not necessarily inorganic, but found that the addition of 5 per cent. sucrose to milk was detrimental to lactation and reproduction, which ceased in the second generation. On the other hand, Krauss, Erb and Washburn(39) report sub-normal growth of rats (as compared with rats on a good mixed diet) on a milk-iron-copper diet.

Considerable attention has recently been paid to soft-curd milk. A Utah *Bulletin* by Hill(47), the pioneer in this field, gives a description of the technique of measuring the hardness of the curd, and discusses the effect of breed and of environmental and hereditary factors on the curd tension. Most of the papers deal with clinical aspects of the problem. Espe and Dye(48), however, studied the effect of the curd tension on the digestibility of milk, using dogs, with Pavlov stomach pouches, as experimental animals. They found that adult dogs required definitely less time to digest low-curd tension milk than milk having a relatively tough curd. The percentage of casein in the milk seemed to be the greatest factor influencing curd tension. This finding was confirmed in an elaborate chemical study of soft-curd milk by Weisberg, Johnson and McCollum(49).

The effect of the level of protein feeding of the cow on the nutritive value of milk has been studied by Krauss and Hayden(16). No difference was found in the total nutritive effect of either "low-protein" or "high-protein" milk supplemented with iron and copper. Similarly the biological value of the mixed proteins of milk was

unaltered by the difference in feeding. Nevens and Shaw(50) found no difference in the apparent digestibility of the fat, protein, total solids and sugar of the milk of Ayrshire, Brown Swiss, Holstein and Jersey cows.

B. *Treated milk.*

(*a*) *Effect of heat.*

(i) *General.* The problem of heat treatment of the liquid milk supply has been very much in the foreground as evidenced not only by the number of original papers but also by the various reviews and summaries which have appeared within the last two or three years. In this connection the comprehensive survey of experimental and clinical evidence by Stirling and Blackwood(51), the review of Savage(52), that of Krauss, Erb and Washburn(39), and that of Isaachsen(53) should be consulted.

Of the original papers a large number deal with the effect of heat treatment on various individual nutritional factors in milk; several, however, are devoted to a comparison of the general dietary properties of heated and raw milk.

The work of Scott and Erf(37) has already been mentioned in connection with the discussion of the nutritional properties of raw milk. In feeding experiments on rats these workers have compared raw certified milk, raw milk from specially fed cows (a special mineral mixture and dry fish meal being given in addition to the usual grain mixture and alfalfa hay) and commercial pasteurised milk. The milks constituted the sole diet of the rats and the first two were found to be greatly superior to the heated milk both in their anti-anaemic and growth-promoting properties. The rats on heated milk were sterile, while several litters were born in the raw-milk groups. Unfortunately, the rather elementary precaution of using milk from the same source for the "raw" and "heated" groups had not been observed. Later this essential condition was fulfilled and the differences (in the rate of prevention of anaemia and growth) in favour of raw milk, while very definite, were smaller than in the previous series. The special pasteurised milk was far superior to that commercially pasteurised. The loss of anti-anaemic properties after pasteurisation noted by Scott and Erf is very difficult to understand in the light of our present knowledge of experimental anaemia, this disease being definitely associated with a deficiency of iron and copper in the milk. The process of pasteurisation would be expected to increase the concentration of these two metals in milk. Krauss, Erb and Washburn(39), who attempted to repeat the experiments of Scott and Erf, compared the raw milk produced on the farm of the Ohio Agricultural Experiment Station with the same milk pasteurised in the laboratory, and also milk from the Ohio State University herd with milk from the same source commercially pasteurised. In their experience anaemia developed on both raw milks and on the respective pasteurised milks, and the heat treatment had no effect on the rate of development. In this case the iron and copper content of the milk was unchanged by the treatment. When the milks were fortified with iron and copper, the total nutritive effect of the raw and treated milk was found to be practically the same when judged by the standard of growth of rats in individually controlled (paired feeding) experiments. Kitchin and McFarland(54) report that rats receiving raw milk *ad lib.*, supplemented by 25 g. of raw carrot per week per rat and water, grew better than rats receiving a similar diet containing milk from the same source but pasteurised (method not stated). Only five rats were started in each group and only three and four of them, respectively, completed the experiment. It is difficult to attach much importance to the result. E. C. V. Mattick and Golding(55) have compared in prolonged experiments the relative values (for growth and reproduction) of raw milk from the University of Reading herd with milk from the same supply pasteurised in the laboratory. The milks were given to the rats *ad lib.*, and they received in addition a limited quantity of

biscuit made from white wheat flour and water. On this diet both groups of rats grew and reproduced, but reproduction and lactation were better on the raw milk. The authors suggest that vitamin B_1 was one of the factors impaired in the course of pasteurisation. These experiments have been repeated by Drummond(56) on rats, and by Wilson and Cowell(57) on mice. Drummond, using approximately the same technique as Mattick and Golding (his milk and biscuit were from a different source), was unable to confirm the findings of these authors as, although his animals produced litters on both diets, none of the young were weaned. He noticed, however, that the animals on raw milk grew better than those on pasteurised. In a further experiment he used milk from the same source as that used by Mattick and Golding, with biscuit made from the same flour. He also again tested milk from his original London supply with his original biscuit. The milks were fed raw and all possible combinations of milks and biscuits were tried, but none of them allowed satisfactory breeding. In a third series of experiments, the combination of milk (raw and pasteurised) and biscuit was fed again to groups of animals, either alone or supplemented with a yeast extract, iron and copper. It was again found that reproduction was unsatisfactory on the unsupplemented diet of milk (raw or pasteurised) and biscuit, but that addition of metals and especially of vitamin B_1 improved it considerably. On the other hand, the weight record of Drummond's animals shows a slight but definite difference in favour of raw-milk animals in all the groups.

Very similar experiments were carried out by Wilson and Cowell(57) on mice. These authors also had to resort to the addition of a mineral mixture and a source of vitamin B to their basal diet of pasteurised or raw milk and biscuit in order to obtain satisfactory reproduction. When this was done, it was found that there was no marked difference in the general development, survival time, reproduction and lactation of mice placed half-grown on either of the supplemented diets, but that litters produced by such mice on raw milk were very definitely heavier at weaning and looked better than similar litters produced on the pasteurised milk diet. These results, dealing with the effect of pasteurisation on the general nutritive properties of milk, all point to some impairment in its nutritive efficiency when tested on rats and mice. The nature of this impairment is rather obscure. The experiments of Mattick and Golding, Drummond and Wilson and Cowell would point in the direction of vitamin B_1. That this factor is reduced in the course of pasteurisation has also been demonstrated by Krauss, Erb and Washburn(39), though these authors believe that the loss of about 20 per cent. potency of the milk in this respect would be more than covered by the margin of safety afforded by the large amount of milk consumed by rats on an exclusive milk diet. It might well be argued, however, that the partial loss of potency, while of no importance for growth alone, would be evident in times of stress such as is caused by the increased requirements of lactation for vitamin B_1.

(ii) *Vitamins.* Spruyt and Donath(58), in experiments on the tropical bird *Munia Maja*, found no loss in the antineuritic vitamin content of milk even after air had been passed through it for 1 hour at 100° C. According to Krauss, Erb and Washburn(39), vitamin B_2 is not affected by laboratory pasteurisation.

There is unanimity of opinion with regard to vitamin C, all authors agreeing that the content of this factor is definitely decreased during pasteurisation. Thus Schwartze, Murphy and Cox(59) state that aerobically pasteurised milk may lose from 20 to 40 per cent. of its original vitamin C potency when the process is carried out in aluminium vessels, but that the loss is higher in tinned copper vessels, amounting to 80–90 per cent. when naked copper is used. Similar observations are reported by Schieblich(60). Kieferle and Eisenreich(24) have studied, both in animal experiments and by means of the chemical test, the effect of various methods of pasteurisation on the vitamin C content of milk. Guinea-pig tests showed that all methods

of heating were detrimental to the antiscorbutic properties of milk, but that flash methods were definitely less harmful than holder methods. The least destruction followed simple boiling. Chemical tests of these milks, which have also been in part reported by Schlemmer, Bleyer and Cahnmann(25), yielded on the whole similar results, though the agreement between both groups of workers is not very good. It should be noted that it is very doubtful whether the rat requires vitamin C at any stage of development. These findings, therefore, do not throw light on the nature of the differences between raw and pasteurised milk as demonstrated in rat-feeding experiments.

According to Krauss, Erb and Washburn(39), the vitamin A content of milk is not affected by pasteurisation. On the other hand Schmidt-Nielsen, Flood and Stene(61) report that pasteurised, and to a lesser extent stassanised, milks lose some of their original vitamin A activity. It would seem, however, that the differences are so small that they would hardly warrant such conclusions, especially in the case of the flash pasteurised milk. Lundborg(62) has attempted to resolve the point by applying the antimony trichloride test to butter churned from raw and from laboratory pasteurised milk. His conclusion that pasteurisation causes a loss of vitamin A is, however, hardly justifiable, as he used single samples of each butter, only one of which was saponified.

The relative resistance of vitamin D to heat treatment would indicate that it is unlikely to be damaged in the course of pasteurisation. The findings of Krauss, Erb and Washburn(39) confirm this view. These authors found no difference in the vitamin D activity of butter churned from either pasteurised or raw milk. They also found that bones of rats subsisting solely on raw or pasteurised milk fortified with iron and copper were equally well calcified. On the other hand, Ellis and Mitchell(63) have found in carefully controlled experiments that, when rats are restricted to suboptimal quantities of calcium derived mainly from an addition of either raw or pasteurised milk to a low calcium diet (containing a sufficiency of vitamin D), the calcium of raw milk is definitely better utilised than that of laboratory pasteurised milk. This was demonstrated by metabolic experiments and by an analysis of the carcases. The efficiency of utilisation of calcium was very high in both groups, being computed at 98 per cent. for raw milk and 92 per cent. for pasteurised milk. Ellis and Mitchell found it difficult in the light of present knowledge to account for the lowered utilisation of the calcium of pasteurised milk. In view of their previous experience they doubt whether the slight chemical changes in the composition of the calcium and phosphorus salts of milk known to take place as a result of pasteurisation can appreciably alter the value of calcium for growth. They suspect a vitamin-like factor to be at issue, and suggest that as vitamin C is the only recognised factor known to be definitely impaired by pasteurisation, it may yet be found that it plays a rôle in the nutritive economy of the rat. It may be mentioned here that Hess and Benjamin(64) studied the various forms of calcium and phosphorus in milk and found that pasteurisation had no effect on the partition either of calcium or of phosphorus. According to Catel(65), kids often thrived better on quickly boiled than on raw cow's milk.

(iii) *Other factors.* In addition to animal work several authors have attempted to study in experiments *in vitro* the nutritional changes following pasteurisation. Andross(66) has studied the peptic and tryptic digestion of certified and pasteurised milk. Taken at their face value these experiments would demonstrate a very inferior digestibility of pasteurised milk. Unfortunately the raw milk was derived from a different source than the pasteurised, and the comparison is invalid. Lautz(67) found that the peptic digestibility of milk was unaffected by heating. Miyawaki, Kanazawa and Kanda(68) found very marked differences in experiments *in vitro* on the digesti-

bility of milks dried by different methods, but the differences proved to be negligible when *in vivo* tests were carried out on rats. The peptic digestibility of raw milk was found to be definitely lower than that of three out of four tested brands of dried milk. On the other hand Nevens and Shaw (69) found in experiments on rats that the apparent digestibility of the proteins of whole milk was higher than that of two powdered milks. It is again unfortunate that the raw and treated milks were from different sources. In another paper these authors (70) report a comparison of milk from the same source before and after evaporation and found that evaporation in glass vessels lowered the apparent digestibility. On the other hand the total nutritional effect was better with evaporated milk than with the corresponding raw milk when fed as a sole diet. The authors suggest (71) that the main reason for the superiority of some brands of evaporated milk over whole milk is the presence of traces of minerals added during the evaporating process, while the greater concentration of nutrients in evaporated milk may also contribute to the observed effect. All these results are very conflicting, and it must be said that most students of nutrition are of the opinion that it is exceedingly doubtful whether *in vitro* experiments are of any value even as a rough guide to the actual performance of the test substances in the living organism.

(*b*) *Irradiated, vitaminised and mineralised milk.*

Attempts to bring the vitamin A concentration of winter milk and butter up to the summer level are discussed in section C (Milk Products). It is at present generally agreed that even the best pasture-produced summer milk is deficient in several respects, if it constitutes, for any length of time, the sole diet of infants. Of these deficiencies the most prominent is the low concentration of the antirachitic factor. In the years covered by this review attempts to alter the composition of milk, either by special feeding of the cows or by subsequent treatment of the milk, have been especially numerous. The concentration of some factors in milk can be readily altered by suitable feeding, other factors are under physiological control. The possibility of altering the composition of milk has been discussed by Hart (72). It seems reasonable to expect that those factors, which in any case are subject to seasonal variation, will be most easily affected. By far the largest number of papers, chiefly of American origin, deal with methods of increasing the vitamin D content of milk. The process of irradiating milk with ultra-violet rays, which had been extensively used in Germany for the last few years, has been very carefully studied in the laboratory and clinically by Supplee, Hess and their collaborators (73, 74, 75, 76, 77, 78, 79). These authors used powerful carbon arcs of the flaming type and irradiated the milk (1·2 per cent. fat) for a very short time (not more than 16 sec.) in a thin film under very carefully controlled conditions. They found that the application of about 2,500,000 ergs per ml. produced the maximum concentration of vitamin D. They produced in this way milk uniformly possessing an activity of about 50 Steenbock units per quart, or approximately 12 times the amount present in the original milk. (1 Steenbock unit is equal to about 2·7 international units.) This milk when pasteurised, boiled, or dried after irradiation, has been found, in extensive tests (which are reviewed on p. 264) to be clinically active. Similar laboratory observations have been reported by Steenbock (80). Anderson and Triebold (81) have studied the biochemical changes which take place in milk after ultra-violet irradiation, and in butter churned from this milk. They found very slight changes after a heavy dosage (8 times the normal exposure) of ultra-violet light, and they conclude that no change would be noticeable after normal irradiation. Supplee and Dow (82) found that irradiation of liquid milk for a few seconds caused a slight but definite destruction of the anti-scorbutic vitamin, while no change was noted in dried milk. According to Bender

and Supplee(83) irradiation up to 48 sec. (up to 6,780,000 ergs per ml. of milk) did not affect the vitamin A potency of milk. Mitchell, Eiman, Whipple and Stokes(84) irradiated whole milk by Supplee's method and found no loss in vitamin A or C. The milk had a potency of 65 Steenbock units per quart.

Irradiated yeast is now extensively fed to cows as a means of enhancing the vitamin D content of milk. The technique of this method, which has been used for some time in America, has been worked out in considerable detail by another large team of workers(85, 86, 11, 87, 88). The general plan of this series of experiments has been described by Hardenbergh and Wilson(89). It has been found that about 60,000 Steenbock units of vitamin D have to be fed daily to a cow in order to produce a milk of the requisite clinical potency, namely 150–160 Steenbock units per quart(86). (The difference between irradiated milk and "yeast milk" in this respect is discussed on p. 265.) The amount of yeast required varied according to the milk yield of the cow, and it was found(87) that heavy milkers were more efficient in transferring the vitamin D from the food to the milk than poorer milkers. It is pointed out by Russell(88) that it takes about three weeks from the beginning of the yeast feeding for the milk to reach its maximum potency, which thereafter is steadily maintained. Only about 2·3 per cent. of the vitamin D fed is recovered in the milk and about 30 per cent. is excreted in the faeces(11). About 70 per cent. of the vitamin D fed may be demonstrated in the blood in the first and second hours after feeding, but the concentration in the blood subsequently drops very rapidly, and, most probably, the vitamin D undergoes destruction in the blood stream(87). The feeding of moderate amounts of irradiated yeast, as used in the commercial production of the yeast milk, had no effect on the chemical composition of the milk. When excessive quantities were fed (about 10 times the usual dosage), a slight rise in the Ca/P ratio and ash content of the milk was noted(11).

Other highly active substances have also been fed to cows to increase the potency of the milk. The addition of irradiated ergosterol was studied by Hess, Lewis, MacLeod and Thomas(86), by Thomas and MacLeod(85), and by Krauss, Bethke and Munroe(90). The two former authors(85, 86) found that it was necessary to feed to the cow about three times as many Steenbock units in the form of irradiated ergosterol as in the form of irradiated yeast in order to produce milks having the same final rat potency. Krauss, Bethke and Munroe(90) state that the feeding of irradiated ergosterol is not economical at the present price of the preparation. In the opinion of Krauss, Bethke and Wilder(91) the same applies to the feeding to cows of a concentrate from cod-liver oil prepared by the method of Zucker. It was found necessary to feed 60,000 rat units in this form to produce an elevenfold increase in the vitamin D content of milk (from 2·8 to 30 Steenbock units per quart). Laquer(92) and Shelling and Tidwell(93) advocate the direct addition of irradiated ergosterol to milk in order to increase its vitamin D content. Zucker(94) describes a method of adding his concentrate to milk. This method has found practical application in the United States, and a dairy has been licensed for the sale of "vitamin D concentrate milk." Yet another method of producing antirachitically potent milk, that of irradiating cows with carbon arc lamps, is dealt with on p. 265.

The revival of interest regarding the well-known paucity of milk in iron and the newly acquired knowledge of the essential nature of copper, in which milk is also deficient, have led to various suggestions that liquid milk should be "mineralised" by direct addition of the missing metals. "Iodisation" via the cow has also been suggested. (The level of iodine in milk is very definitely related to that of the feed.) Such tampering with milk is unanimously condemned by research workers and medical bodies(95, 96, 97, 98).

C. *Milk products (butter). Vitamins.*

Vitamin A. It has been suspected for a long time, but only recently demonstrated with certainty, that the yellow pigment carotene may be transformed in the animal body into the colourless vitamin A. There is no vitamin A in the vegetable foods forming the bulk of the cow's ration, but it has been shown by Moore(99) that she is able to transform the pigment into vitamin A. Morton and Heilbron(100) were the first to demonstrate the presence of these two substances in butter. A biological vitamin A assay measures simultaneously both these substances in proportion to their relative vitamin A activities. Unfortunately all biological methods of vitamin assay are time consuming and fraught with difficulties, and this is especially so in the case of vitamin A. It is therefore gratifying that both carotene and vitamin A can at present be measured by physical and chemical methods. It should be noted that physical measurements, though giving accurate values for the quantities of vitamin A and carotene present, cannot yet be directly translated into biological units because of the uncertainty as to the relative biological activity of carotene and vitamin A *sensu stricto*. The methods are applicable to butter, and it seems that a spectrophotometric measurement of the absorption at 328 mμ for vitamin A, and at about 460 mμ for carotene yields true figures with regard to their concentration in butter(101, 102). According to Shrewsbury and Kraybill(103) a colorimetric estimation of carotene in butter gives a result several times too high, although when butter is dissolved in petroleum ether satisfactory readings can be obtained. Strictly speaking the biologically inactive xanthophyll should be removed before carotene is measured by physical methods, but Gillam(104) has recently suggested a simplified method, based on the constancy of the ratio of carotene to xanthophyll in butter, which dispenses with this tedious separation. The well-known Carr-Price antimony trichloride reaction cannot be applied directly to butter because of the presence in it of an inhibitory substance which, moreover, shows seasonal variation, being present in greater concentration when the cows are at pasture than when they are stall fed(105). For this reason values reported by Frengley and Herrick(106) for the vitamin A content of New Zealand butter are unfortunately valueless. It is stated by Gillam(104) that even after saponification of the butter the spectrophotometric measurement of the blue colour given by the Carr-Price reagent yields lower vitamin A values than those measured by the absorption at 328 mμ.

Whether employing biological or physical methods of measurement, investigators have lately been especially interested in the effect of the food of the cow on the vitamin A content of milk and butter. The general consensus of opinion is that access to green pasture yields milk and butter of high vitamin A activity, while stall feeding, unless special precautions are taken, results in a low yield of the vitamin. Thus Crawford and her collaborators(107) demonstrated no difference in the vitamin A activity of summer and winter samples of New Zealand butter when the cows had been out on pasture throughout the experimental period. On the other hand, Fraps and Treichler(108) found that cows receiving only cotton-seed meal and hulls produced butter containing only about one-fifteenth of the amount of vitamin A present in butters from cows similarly fed but allowed access to Sudan grass pasture. Baumann and Steenbock(102) have measured by the spectrophotometric methods of Heilbron and Morton the vitamin A and carotene content of butter made from the general supply of milk from the University of Wisconsin Dairy throughout the year, and have found a 400 per cent. variation in the content of carotene and a 100 per cent. variation in the content of vitamin A. The lowest values were found in April butter, and the highest in June and July. At the same time very similar results were reported by Booth, Kon, Dann and Moore(105), Watson, Drummond, Heilbron

and Morton(109), and Gillam, Morton, Heilbron, Bishop and Drummond(101). All these observations bring home the unpleasant truth that the concentration of vitamin A and carotene in butter is at its lowest in winter and early spring, that is, at times of greatest need. It seems, therefore, only natural that attempts have been made to maintain, by suitable feeding, the vitamin A content of butter and milk at the summer level throughout the year. Hilton, Hauge and Wilbur(110) found that good quality alfalfa hay and, to a lesser extent, soya-bean hay, were very effective either in maintaining a high vitamin A level, or restoring it to normal summer values after it had declined under the usual stall-feeding conditions. Similar results were obtained by Meigs, Hartman and Converse(111). Gillam and his collaborators(101) and Watson and his collaborators(109) found that the addition of grass, artificially dried by methods used at the Imperial Chemical Industries Agricultural Research Station, to a normal winter ration, maintained the vitamin A and carotene content of the butter throughout the stall-feeding period almost at summer level. Watson and Ferguson(112) found that the inclusion of artificially dried grass (50 per cent. of the concentrate ration) in the winter ration raised the yellow colour of butter almost to summer level, while the feeding of A.I.V. fodder at the rate of 40 lb. per head per day was even better in this respect.

It is well known that Guernsey and Jersey cows yield butter more highly coloured than that of other breeds, and claims have been advanced, especially since the recognition of carotene as a precursor of vitamin A, that the vitamin A activity of these butters is higher. This is not borne out by experimental evidence. On the contrary, Wilbur, Hilton and Hauge(113) found no difference in vitamin A activity, as measured in biological experiments, between Guernsey and Ayrshire butterfats produced under identical conditions of feeding, though the former contained twice as much carotene as the latter. Kon and Booth(114) made similar observations with Guernseys and Shorthorns. The latter authors also measured the vitamin A content by the antimony trichloride reaction, after saponification of the butters, and found that the Guernsey butter contained less vitamin A than Shorthorn butter though the yellow colouration of the former was three times as intense as the latter. The results of Davis and Hathaway(8), mentioned on p. 251, would on the other hand, tend to indicate differences in the vitamin A content of the butter of various breeds, as their Holstein milk obviously contained much less fat than the Jersey milk but was in their opinion just as active. In their latest publication(115) they state that when fat percentages are equalised Holstein milk is more active than Jersey milk, and the same applies to 20 per cent. cream from both breeds. Baumann, Steenbock, Beeson and Rupel(116) have studied by spectrophotometric methods the influence of breed and diet on the vitamin A and carotene content of the butter.

Vitamin D. The vitamin D content of butter varies with the season in the same manner as the vitamin A content, and it is generally agreed that the concentration is higher in summer than in winter. Crawford and her colleagues(107) found that the vitamin D content of New Zealand winter butter was definitely lower than that of summer butter, though the cows were on pasture all the year and the vitamin A level remained constant. Watson *et al.*(109) found that the feeding to stall-fed cows of nitrogen-fertilised dried grass cut in autumn did not increase the vitamin D content of butter, though the vitamin A content increased to almost summer level. These observations emphasise the significance of the action on the cow of direct or indirect sunlight rather than of the ration itself. The recent work of Bechdel and Borland(117) suggests, in the opinion of the reviewer, that direct insolation of the cow may play a dominant rôle in the formation of the antirachitic substance in milk under normal conditions. It is interesting to note that Sabri and Fikry(118), working in Cairo, were unable to demonstrate the presence of vitamin D in cow's butter or in buffalo milk

fat even when these were incorporated in the 2965 rachitogenic diet of Steenbock to the extent of 12 per cent. On the other hand, Bechtel and Hoppert(119) report an exceptionally high content of vitamin D in a sample of summer butter produced on Sudan grass pasture. Olson(120) studied the seasonal variation of vitamin D in butter. The vitamin D content of summer butter was substantially higher than that of winter butter.

The rat is a most convenient animal for the estimation of the vitamin D values of various substances, but considerable doubt has recently been cast on the validity of such findings when applied to human nutrition. In the first place, the rat is unable to differentiate the vitamin D of cod-liver oil from that of irradiated ergosterol, though both substances differ in their action on the infant(121). In the second place, as pointed out on p. 265, the rat test cannot serve as a guide to the clinical value of various milks where the antirachitic potency has been raised by one of the several methods now available for this purpose. In addition Kon and Booth(122, 123, 124) found in experiments on rats that about 80 per cent. of the original antirachitic activity of autumn and winter butter was lost as a result of saponification, while the activity of cod-liver oil and irradiated ergosterol remained unimpaired under identical conditions of treatment. Summer butter was more resistant, while highly potent butters obtained by feeding irradiated yeast to cows or by irradiation of the butter itself withstood saponification without loss. They suggest that the activity lost in saponification was that due to the fat of butter which affected the calcium and phosphorus metabolism of the rat. It is very doubtful whether the fat moiety of butter will exert an antirachitic action on species in which absence of vitamin D alone leads to the appearance of rickets, independently of the mineral balance of the diet. The possibility is not excluded that the labile activity of butter is in part, at least, due to a genuine labile antirachitic factor. Whatever the ultimate finding may be, all these results indicate the necessity for extreme caution in translating rat values for vitamin D into clinical units.

II. The nutritional value of milk for human beings.

A. *Raw milk.*

Within the years covered by this review many papers have been published which show the undeniably beneficial effects of milk when added to the diet of the average growing infant or child. Only a few of the papers deal with experiments in the exact sense of the word. The majority constitute a survey of dietary habits, but in all of them the opinion is expressed that children receiving milk thrive better for this addition. This experience, gleaned in different countries, demonstrates, as justly pointed out by Stenhouse Williams and E. C. V. Mattick(125), not only the feeding value of milk, but also the fact that in almost all cases the children concerned were receiving an insufficient diet. To the numerous papers published on the subject in previous years the following may now be added: Turbott and Roland(126), working with Maori children found that a group of ninety-three children receiving from $\frac{1}{2}$ to 1 pint of milk daily gained twice as much in height and two and a half times as much in weight as a control group of children not given milk. Rabasse(127) in France found a greater increase in weight in primary school children receiving 250 g. of milk soup than in control children receiving no supplement. Similar results are reported from Japan(128) where, in an investigation carried out in Tokyo schools, children receiving the small allowance of from one-third to two-thirds of a pint of milk daily made quite spectacular increases in weight, and to a lesser extent in height, over the control group. In this country benefit derived from milk meals by children is pointed out

in the *Annual Report of the Chief Medical Officer of the Board of Education* for the year 1931 (129). Lininger (130) reports from the United States results of an extensive study of the effect of the addition of milk to the diet of under-nourished Philadelphia school children who were 13 per cent. or more under weight according to the Baldwin-Wood standards. The gain in weight was definitely correlated with the amount of milk consumed. The large-scale Lanarkshire experiment of Leighton and McKinlay (131) is discussed in detail later. It may be stated here, however, that whatever criticisms have been levelled at the conclusions drawn by the authors, it is agreed that this experiment demonstrated the beneficial effect of the addition of milk to the diet of 10,000 children.

Sprawson (132) showed the great importance of an adequate consumption of milk in preventing the development of dental caries in children. He noticed in 1928, in a residential institution consisting of some 750 boys, a greatly diminished incidence of caries, and learnt afterwards that from 1925 onwards 1 pint of raw milk had been added daily to the institutional diet. On further investigation he was able to correlate the freedom from decay with the consumption of raw milk, and found that out of a group of seventy-three children who started taking raw milk before the age of 2 years and 7 months, sixty-three were totally free from dental caries. The importance of milk as a protective food is demonstrated by M. Mellanby in her monograph on the effect of diet on teeth (133).

Holmes, Pigott, Sawyer and Comstock (134) describe the objective and subjective improvement in the health and efficiency of sixty-three under-weight young women following the addition of 1 pint of milk and 1 tablespoon of cod-liver oil daily to their diet. It may not be out of place here to refer to the recent study of the milk consumption of 1000 families in County Durham by Burns (135) which clearly demonstrates the appallingly low consumption of milk by the poor population of a representative English county.

B. *Treated milk.*

(*a*) *Pasteurised milk.*

The evidence in favour of the dietetic importance of milk is beyond dispute. That dealing with the effect of heat on its nutritional value is much less satisfactory. The appraisal of the difference between raw and pasteurised milk obviously requires a much more delicate technique than the demonstration of the superiority of a diet containing milk over a diet without milk. It may be said that so far no practical experiment on children has been described which would differentiate between raw and pasteurised milk in a way to satisfy the statistician.

In addition to the reviews of Stirling and Blackwood (51) and of Savage (52), the recent *Report of the Committee on Cattle Diseases* (136) gives an authoritative survey of the effect of pasteurisation on the constituents of milk.

No experiment has been more in the foreground in recent years than the large-scale Lanarkshire experiment of Leighton and McKinlay (131). This experiment is too well known to need detailed description here. In addition to numerous commentaries in medical journals it has been discussed in the reviews mentioned above. Briefly stated the experiment was as follows: For 4 months in Lanarkshire in certain schools 5000 children were given $\frac{3}{4}$ pint of raw milk daily and 5000 children in these same schools were used as controls. In a different set of schools 5000 children received $\frac{3}{4}$ pint of the same milk pasteurised, and another 5000 in these schools acted as controls. The children were weighed and measured at the beginning and end of the experiment. In the opinion of the authors the experiment, while demonstrating the beneficial effect of the addition of milk to the diet of school children, showed no difference between the effects of raw and pasteurised milk. This point of view was

challenged by Bartlett(137) and by Fisher and Bartlett(138), who pointed out the unfortunate planning of the experiment, in that raw and pasteurised milk were not compared in the same schools, but stated that one conclusion should have been "That the growth response in height to raw milk is significantly greater than that to pasteurised milk." "Student"(139), after careful investigation, noted two important defects in the Lanarkshire experiment: firstly, the selection of controls was not random, with the result that the control children were on the average taller and heavier than those given milk; and secondly, the weights of the children included some clothing, and the amount discarded between the commencement (February) and end (June) of the experiment varied in the different groups, and probably the "better off" children discarded most clothing in the summer. "Student" concluded that the Lanarkshire experiment failed to produce a valid estimate of the advantage of giving milk to children and of the difference between raw and pasteurised milk. He suggested that, in order to obtain an unequivocal result, either the large-scale experiment should be repeated or a small experiment be run on twins of like sex. In an attempt to approximate such an ideal experiment Elderton(140) selected pairs from the original cards of the Lanarkshire experiment, control and raw milk feeders, and control and pasteurised milk feeders, who had the same initial height and weight within reasonable limits. From statistical investigation of the selected material Elderton came to the conclusion that those who had extra milk gained in height and weight over those who did not, but that there was no evidence that raw milk had an advantage over pasteurised, or pasteurised over raw, in increasing growth when the two were directly compared on this selected material. In the opinion of Elderton, expressed with some finality, the question of the value of pasteurisation thus turns practically on the elimination of possible sources of disease or on determining whether certain cases of disease are less frequent when pasteurised rather than raw milk is taken. The reviewer had the privilege of discussing this paper with "Student"(141) who wrote as follows: "Dr Elderton's evidence as to the value of milk to growing school children must be allowed due weight. As to the question whether raw and pasteurised milk are equally valuable I do not think that Elderton has been able to overcome the original defect of the experiment, namely that the two milks were tried in different schools which may not have been chosen at random; for example, the controls in the schools taking raw milk gained less in weight by an amount almost, but not quite, significant, than those in the schools taking pasteurised milk, as evident from Table 4, p. 330 of Elderton's paper. As the gain of weight is given as nett, *i.e.* after the loss of weight due to the replacement of winter by summer clothing, a small gain in weight indicates a more prosperous state of affairs and therefore a tendency to benefit less by an extra ration of milk. This would tend of course to handicap raw milk in a comparison with pasteurised."

This large-scale experiment thus definitely demonstrates the value of milk as such, but it no more than indicates the possibility of the superiority of raw milk. If an unequivocal answer is required, it will have to be sought in a new experiment. Very much the same applies to the observations of Sprawson(132). While his conclusions with regard to the value of milk are unquestionable it is difficult to accept his evidence that raw milk is far superior to pasteurised. He compares two small groups of children who received 12 oz. of pasteurised milk with or without cod-liver oil in one institution with children who received 20 oz. of raw milk in another institution, and though the differences are marked it seems impossible to accept these results as anything more than an incentive to further investigation. Frank *et al.*(142) have attempted in an extensive field study to compare the relative merits of raw and heated milk. In thirty-nine American cities over 3000 children were weighed and measured. The consumption of milk was recorded and the children were finally

divided into two groups, one group which had received exclusively heated milk (pasteurised, boiled, evaporated or milk powder), the other group children who had received raw milk for more than the latter half of their lives. No difference was found in the average weights and heights of the two groups. Here again it seems obvious that an investigation of this type, giving no information as to the amount of milk consumed, the composition of the remainder of the diet, etc., yields no basis for the direct comparison of raw and heated milk. Morris and Graham(143) carried out metabolic experiments on two apparently healthy infants using boiled and raw milk from the same source, and found that the absorption of nitrogen, fat and minerals was not diminished by the boiling of milk.

The various aspects of heat treatment of milk command at present a great deal of attention. Whatever the answer to a complicated problem involving important issues independent from nutritional considerations, we would do well to bear in mind that raw milk as it comes from the cow is subject to marked seasonal variation in its nutritional qualities. It is unfortunate that in winter and early spring the increased demands of the growing infant and child for certain dietary factors should coincide with the lowest ebb of these substances in cow's milk. It is possible to produce by dietary means winter milk approximating to summer milk in its nutritional qualities and efforts in this direction should not be spared.

(*b*) *Irradiated and vitaminised milk.*

The various types of vitamin D fortified milk already discussed in this review have been subjected to clinical tests. Hess, Lewis, MacLeod and Thomas(86) tested clinically four different types of milk, (i) a milk containing 160 Steenbock units per quart and obtained by feeding to cows 60,000 Steenbock units daily in the form of irradiated yeast; (ii) a milk containing half this potency (from cows receiving 30,000 Steenbock units); (iii) a milk of the same potency as (i) obtained by feeding 200,000 Steenbock units of irradiated ergosterol; and (iv) a milk of half this potency produced by cows receiving 100,000 Steenbock units of irradiated ergosterol. The two higher levels when given in quantities of about $1\frac{1}{2}$ pints daily to groups of twenty-five infants either protected them from X-ray rickets, or cured the condition within a month. The lower levels were not so effective. Clinical experience thus confirms the results of the work carried out by the same authors on rats, namely that it is necessary to feed to cows about three times as many rat units in the form of irradiated ergosterol as in the form of irradiated yeast in order to produce milks of equivalent potencies. Pasteurised "yeast milk" of the potency of 160 Steenbock units per quart was also tested by Wyman and Butler(144) and found to be highly active in curing four cases of definite rickets when given in amounts of 1 quart daily. Five minutes' boiling did not impair the activity of the milk. Gerstenberger and Horesh(145) tested clinically the irradiated ergosterol milk produced by Krauss, Bethke and Munroe(90) and described on p. 258. The milk contained approximately 100 Steenbock units per quart and in amounts of 1 pint daily produced a slow but definite improvement in two rachitic infants.

The clinical value of irradiated milk (p. 257) has been reported on by Supplee, Dorcas and Hess(73) and more fully by Hess and Lewis(76). The pasteurised milk was fed to a group of about 100 babies, including many negroes (who are especially susceptible to rickets), and gave very good results in quantities of about 750 to 1000 ml. milk daily, *i.e.* not more than 50 Steenbock units. No direct comparison with the "yeast milk" was made, but Hess and Lewis state that this treatment was at least as efficacious as that with "yeast milk" containing 160 Steenbock units per quart. This work has been criticised by Kramer and Gittleman(146), who tested clinically irradiated milk at two levels of intake, namely 55 and 40 units, and two "yeast

milks" of similar potencies, each level contained in 1 quart of milk. They found irradiated and yeast milk to be equally effective and active even at the remarkably low level of intake of 40 Steenbock units daily. In a subsequent paper Hess and Lewis(147) compared irradiated milk, "yeast milk," cod-liver oil, and viosterol (irradiated ergosterol) in their ability to bring about a cure of X-ray rickets, and found that the same degree of cure was brought about by 35–42 Steenbock units daily in the form of irradiated milk, about 70 units in the form of "yeast milk," 250 units in the form of cod-liver oil, and from 600 to 800 units as viosterol. This confirmed therefore the previous contention that irradiated milk is twice as potent as yeast milk, though their rat potencies may be equal. Mitchell, Eiman, Whipple and Stokes(84) gave infants irradiated milk and also milk from cows whose udders and undersides had been irradiated with powerful carbon arcs. Only one level of each milk was tested. Pasteurised irradiated milk containing 65 Steenbock units per quart was found to give good protection or cure, and a similar result was obtained with the milk from irradiated cows containing the astonishingly small amount of 22 Steenbock units per quart. On the other hand, Wilson(148) found that the administration of milk containing 150 Steenbock units of the Zucker cod-liver oil concentrate (discussed on p. 258) per quart was unable to prevent the appearance of two cases of moderate rickets and seventeen cases of mild X-ray rickets in thirty-three infants who started receiving the milk when under 8 weeks old and were under observation from 3 to 6 months. The children consumed from 100 units daily at the beginning of the experiment, to about 140 units at the end. The milk mixtures were cooked in a double boiler for 20 min. These findings would confirm the opinion of Hess and Lewis(147) that when equal rat units are administered, irradiated milk and yeast milk are more efficient than cod-liver oil, though the rather prolonged boiling may have affected the results. Although for obvious reasons clinical tests are less quantitative than laboratory experiments on animals, it seems reasonably certain that the number of rat units contained in a fortified milk cannot serve as a guide to its clinical value.

Yeast milk has also been given to lactating mothers, and Bunker, Harris and Eustis(149) found, in experiments on rats, a definite increase in the antirachitic potency of milk of mothers who had received daily 120 Steenbock units of vitamin D contained in 1 quart of yeast milk. McBeath and McMahon(150) gave a quart of yeast milk (160 Steenbock units) to expectant mothers from about the sixth month of pregnancy. The babies (sixteen in number) were observed from 2 months of age onwards and were compared with a group of twenty-six babies whose mothers were getting 1 quart or ordinary milk. The incidence of rickets was found to be much smaller in the first group than in the second, though many of the control cases were given some form of antirachitic treatment.

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